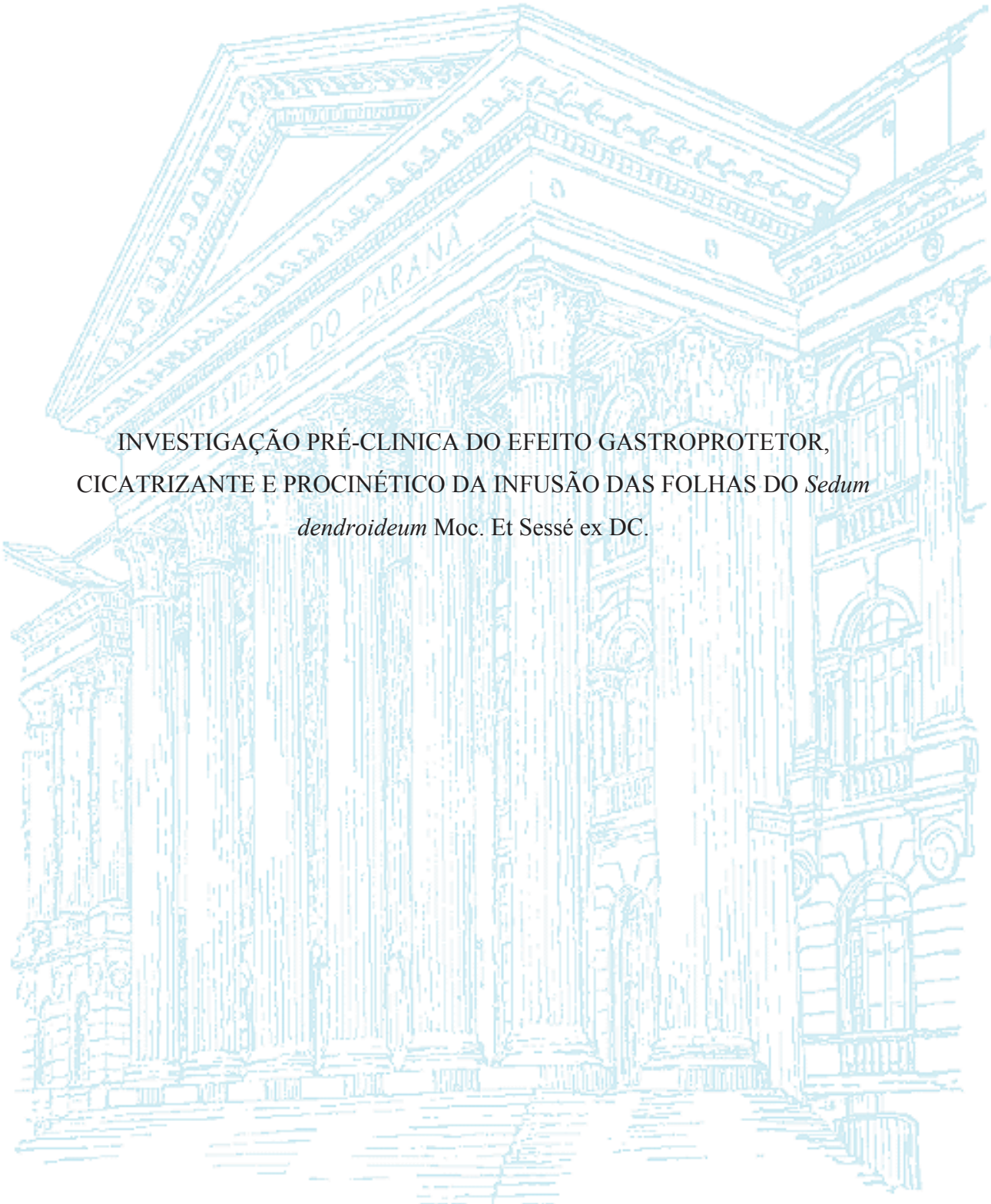
UNIVERSIDADE FEDERAL DO PARANÁ

BRUNA BARBOSA DA LUZ



INVESTIGAÇÃO PRÉ-CLINICA DO EFEITO GASTROPROTETOR, CICATRIZANTE E PROCINÉTICO DA INFUSÃO DAS FOLHAS DO *Sedum dendroideum* Moc. Et Sessé ex DC.

CURITIBA
2018

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INVESTIGAÇÃO PRÉ-CLINICA DO EFEITO GASTROPROTETOR, CICATRIZANTE E PROCINÉTICO DA INFUSÃO DAS FOLHAS DO *Sedum dendroideum* Moc. Et Sessé ex DC.

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Orientadora: Profa. Dra. Maria Fernanda de Paula Werner

Co-orientadora: Dra. Daniele Maria Ferreira

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **BRUNA BARBOSA DA LUZ** intitulada: **Investigação pré-clínica do efeito gastroprotetor, cicatrizante e procinético da infusão das folhas de *Sedum dendroideum* Moc. et Sessé ex DC**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 19 de Junho de 2018.

MARIA FERNANDA DE PAULA WERNER
Presidente da Banca Examinadora (UFPR)

ODERY RAMOS JUNIOR
Avaliador Externo (UFPR)

CLELIA AKIKO HIRUMA
Avaliador Externo (UNESP)

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RESUMO

Sedum dendroideum Moc *et Sessé* ex DC, popularmente conhecido como Bálsamo é uma espécie de planta suculenta da família Crassulaceae, utilizada na medicina tradicional para o tratamento de inflamações, cicatrização e gastrite na forma de infusos, chás ou salada fresca. O estudo teve como objetivo avaliar o efeito gastroprotetor, cicatrizante gástrico e sobre a motilidade gastrointestinal, bem como os possíveis mecanismos de ação envolvidos. A caracterização bioquímica do infuso preparado com as folhas do *S. dendroideum* (SDI) demonstrou um extrato rico em compostos fenólicos e flavonoides, tendo sido identificados metabolitos secundários como glicosídeos, incluindo kaempferol, quercetina e miricetrina. A administração oral do SDI exibiu efeito gastroprotetor no modelo de lesão aguda induzida por etanol e no modelo de lesão induzida por indometacina em ratos. Tal efeito envolve o reforço das barreiras protetoras gástricas, incluindo a camada de muco, glutationa (GSH) e óxido nítrico (NO) endógeno. A citoproteção também foi observada por via intraperitoneal, descartando a possível formação de barreira física sobre a mucosa gástrica. Em animais induzidos à hipersecreção, SDI não foi capaz de alterar o volume, pH e a acidez total do conteúdo gástrico, descartando a redução da secreção ácida como seu mecanismo de proteção. O SDI também acelerou a cicatrização de úlceras gástricas crônicas induzidas por ácido acético, acompanhado por aumento no número de células em proliferação e conteúdo de mucina gástrica. O tratamento subcrônico com SDI também diminuiu parâmetros inflamatórios no estômago, como mieloperoxidase (MPO), fator de necrose tumoral (TNF-α), interleucina 1 beta (IL-1β). Ademais, o SDI apresentou atividade antioxidante *per se* no ensaio *in vitro* de DPPH, e em modelos animais demonstrou atividade antioxidante, aumentando os níveis de glutationa (GSH), atividade da enzima catalase (CAT) e superperóxido dismutase (SOD). O tratamento oral com o SDI também exerceu efeito pró-cinético, sem alterar o esvaziamento gástrico. Durante a exposição subcrônica, o SDI não apresentou sinais de toxicidade, nem alterou o peso (corporal e dos órgãos) e parâmetros bioquímicos, como alanina aminotransferase (ALT), aspartato aminotransferase (AST), creatinina e ureia. Coletivamente, nossos resultados demonstraram que o SDI possui efeito gastroprotetor e cicatrizante gástrico, sendo uma alternativa terapêutica promissora e acessível para o tratamento da úlcera gástrica.

Palavras chaves: *Sedum dendroideum*; infusão; bálsamo, úlcera gástrica, gastroproteção, cicatrizante

ABSTRACT

Sedum dendroideum Moc *et Sessé* ex DC popularly known as "bálsamo", is a succulent plant widely used ornamentally. In folk medicine is commonly used for the treatment of inflammations, wound healing and gastrointestinal disorders. The ethnopharmacological use involves the consumption of fresh leaves as salad or an infusion prepared by soaking the leaves in hot water, biochemical characterization demonstrated that the infusion is rich in phenolic compounds and flavonoid contents like quercetin, myricetin and kaempferol, and their glycosides. The oral treatment with *Sedum dendroideum* infusion (SDI) showed gastroprotective effect, preventing the depletion of the gastric mucus layer and glutathione levels (GSH) in the acute ethanol induced ulcer model. The gasprotective effect was maintained when the infusion was administered intraperitoneally, discarding the possibility of the gastroprotective effect of the infusion occurs due to mechanical barrier formation. The SDI was also able to protect the gastric mucosa in gastric lesions induced by non-steroidal anti-inflammatory. Besides, the oral treatment with SDI during 5 days accelerated the healing of gastric ulcers in the chronic ulcer model induced by acetic acid, accompanied by an increase in the number of proliferating cells and the gastric mucin content. Chronic treatment also decreased inflammatory factors such as myeloperoxidase (MPO), tumor necrosis factor (TNF-α), interleukin 1-beta (IL-1b) and antioxidant parameters such as GSH, catalase (CAT) and superperoxide dismutase (SOD). Furthermore, it demonstrated antioxidant activity per se in the in vitro DPPH assay. The oral treatment with the infusion also had a pro-kinetic effect, without altering gastric emptying. And during subchronic oral exposure the infusion did not show any clinical or behavioral changes, body and organ weight was maintained, and biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and urea were not altered, evidencing the possible safety of infusion prepared with the leaves of the Sedum dendroideum.

Keywords: *Sedum dendroideum*; tea infusion; bálsamo, gastric ulcer, gastroprotection, healing

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ARTIGO CIENTÍFICO 2

LISTA DE ABREVIATURAS E SIGLAS

AINEs, Anti-inflamatórios não esteroides;
bFGF, Fator de crescimento fibroblástico;
CAT, Catalase;
DNA, Ácido Desoxirribonucleico;
DPPH, 2,2-difenil-1-picrilhidrazil;
EGF, Fator de crescimento epidérmico;
eNOS, Óxido nítrico sintase;
FDA, Food and Drug Administration;
GPx, Glutathione peroxidase;
GSH, Glutationa;
GST, Glutationa S-transferase;
HGF, Fator de crescimento de hepatócito;
IL-1α, Interleucina 1 alfa;
IL-1β, Interleucina 1 beta;
NO, Óxido nítrico;
PTF, Produto tradicional fitoterápico;
RENAME, Relação Nacional de Medicamentos Essenciais;
ROS, Espécies reativas de oxigênio;
SOD, Superperóxido dismutase;
SUS, Sistema único de saúde;
TNF-α, Fatores de Necrose Tumoral Alfa;
VEGF, Fator de crescimento endotelial vascular;

ARTIGO CIENTÍFICO 1

AA, Ascorbic acid;
AlCl3, Aluminum chloride;
API, Atmospheric pressure ionization;
COX-1, Cyclooxygenase-1;
COX-2, Cyclooxygenase-2;
DPPH, 2,2′-diphenylpicryl hydrazyl;
DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid);
GSH, Glutathione;

HPLC, High performance liquid chromatography;

L-Arg, L-Arginina;

L-NAME, N(ω)-nitro-L-arginine methyl ester;

LC-MS, Liquid chromatography coupled mass spectrometry;

MBM, Municipal Botanical Museum;

MeOH, Metanol;

MS, Mass spectrometry;

NaCO3, Sodium carbonate;

NaOH, Sodium hydroxide;

NO, Nitric oxide;

NSAIDs, Non-selective nonsteroidal anti-inflammatory drugs;

PGE2, Prostaglandin E2;

PPIs, Proton pump inhibitors;

PQD, Pulsed quantum dissociation;

ROS, Reactive oxygen species;

SDI, *Sedum dendroideum* infusion;

TIC, Total ion current;

ARTIGO CIENTÍFICO 2

ALT, Alanine transaminase

AST, Aspartate aminotransferase;

BSA, Bovine serum albumin;

CAT, Catalase;

DAB, 3,3'-diaminobenzidine;

EDTA, Ethylenediaminetetraacetic acid;

HCL, Hydrochloric acid;

HE, Hematoxylin/eosin;

HRP, Horseradish Peroxidase;

HTAB, Hexadecyltrimethylammonium bromide;

IL-1β, Interleukin 1- beta;

MPO, myeloperoxidase;

MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide;

NAG, N-acetylglucosaminidase;

PAS, Periodic acid–Schiff;

PBS, Phosphate buffered saline;

PCNA, Proliferating cell nuclear antigen;

S.E.M, Standard error of the mean;

SDI, *Sedum dendroideum* infusion;

SOD, Superoxide dismutases;

TMB, 3,3′,5,5′-Tetramethylbenzidine:

TNF-α, Tumor necrosis factor alpha;

SUMÁRIO

1. INTRODUÇÃO

1.1. PRODUTOS NATURAIS

Indiscutivelmente, a natureza é uma das principais fontes de compostos químicos biologicamente ativos. A partir de sua observação e compreensão diversas moléculas terapeuticamente benéficas foram extraídas e purificadas contribuindo para a descoberta de novos medicamentos. Por milhares de anos os produtos naturais têm sido utilizados na forma de chás, tinturas, emplastos entre outras formulações farmacêuticas, até que mais recentemente, com o avanço da ciência, foi possível o isolamento e a utilização de compostos específicos (BALUNAS; KINGHORN, 2005). Um exemplo bem conhecido é a morfina, primeiro composto farmacologicamente ativo, isolado e caracterizado a partir da *Papaver somniferum* em 1800, e que é empregada até hoje para o alívio da dor, mas que antes era utilizada na forma de extrato bruto. Ainda, outros compostos úteis para o tratamento da dor, como a codeína e tebaína foram isolados da mesma planta (RAFFA et al., 2018; NAMAN et al., 2017).

Tais descobertas inspiraram a busca por novos extratos e compostos que pudessem ser utilizados para o cuidado da saúde. O aumento do número de pesquisas com plantas que possuem potencial farmacológico é evidente e estima-se que o estudo de plantas medicinais aumenta em torno de 8% ao ano. De fato, em uma meta-análise, Newman e Cragg (2016) demonstraram que 49% dos fármacos aprovados pelo FDA (do inglês *Food and Drug Administration*) entre 1981 até 2014 foram moléculas que tiveram como fonte produtos naturais ou então diretamente derivadas de compostos naturais previamente isolados. Dessa maneira, e baseado nos fatos, as plantas apresentam uma grande contribuição para o desenvolvimento de novos produtos terapêuticos devido aos inúmeros compostos bioativos, sendo uma das fontes mais importantes de busca por novas alternativas na pesquisa farmacêutica (XI *et al.*, 2017). Além disso, os produtos naturais possuem algumas vantagens em relação aos compostos sintéticos, uma vez que seus compostos bioativos podem ser substratos ou então carregados por transportadores do organismo, proporcionando para esses compostos naturais maior biodisponibilidade e consequentemente maior chance de exercer efeitos farmacológicos (HARVEY et al., 2015).

Os compostos orgânicos encontrados nas plantas são provenientes do metabolismo primário e secundário. Os metabólitos primários, como por exemplo, os

polissacarídeos, têm função estrutural, plástica, de armazenamento de energia, assimilação de nutrientes, crescimento e desenvolvimento. Por sua vez, os metabólitos secundários, que são formados a partir dos metabólitos primários, são compostos altamente variáveis de acordo com a espécie e localização geográfica da planta e tem função ecológica, como por exemplo, prevenir a herbivoria ou infecções por patógenos. São exemplos de metabólitos secundários os flavonoides, compostos fenólicos, taninos entre outros (BAXTER et al., 1998).

O efeito farmacológico exibido por estes metabólitos isolados em diversas doenças é notório. Em relação aos metabólitos primários, os polissacarídeos purificados de plantas emergiram como uma importante classe de produtos naturais em consequência à diversidade de suas atividades biológicas, como atividade antitumoral, anti-inflamatória, antioxidante e gastroprotetora (LI et al., 2018; BATISTA et al., 2014, CHEN et al., 2018, DE OLIVEIRA et al., 2018). Por outro lado, os metabólitos secundários isolados como flavonoides também já demonstraram atividades biológicas com grande relevância farmacológica, entre elas: atividade antioxidante, antialérgica, antiviral e gastroprotetora (SOHN et al., 2018; MAKINO et al., 2013; WONG et al., 2017; ALKUSHI; ELSAWY, 2017).

Apesar de o efeito desses compostos isolados em diversas disfunções serem bem descritos na literatura, muitas vezes extratos, infusões ou decocções preparadas de plantas demonstram atividades biológicas que não são observadas com compostos isolados (CASANOVA; COSTA, 2017). É possível que tal efeito esteja relacionado pela ocorrência de sinergismo entre os componentes presentes no extrato, que podem atuar em diversos alvos simultaneamente, auxiliar na solubilidade e consequentemente absorção do extrato ou até mesmo diminuindo o efeito adverso que poderia ser causado por algum dos compostos isoladamente (WAGNER, 2011). Nos últimos anos a sinergia entre diferentes plantas tem sido explorada na clínica para o tratamento de várias doenças, sendo essas combinações equivalentes a tratamentos de referência ou ainda com benefícios adicionais, como a baixa toxicidade e menor número de efeito colaterais. Como exemplo, podemos citar o fitocomplexo Iberogast®, produzido na Alemanha desde 1961 pela empresa farmacêutica Medical Futures Inc. a partir da mistura de nove plantas para o tratamento da síndrome do intestino irritável e outros distúrbios gástricos. Neste caso, as pesquisas demonstram que os efeitos dessas plantas isoladas não são tão pronunciados como quando utilizados em conjunto, demonstrando assim o sinergismo existente entre esses compostos (WAGNER, 2006).

Outro aspecto importante envolvendo o uso de produtos naturais é o aumento do consumo de alimentos funcionais ao longo dos últimos anos. Estima-se que o mercado dos alimentos funcionais movimenta cerca de 60 bilhões de dólares por ano (EUSSEN et al., 2010), apoiado por estudos que associam o potencial terapêutico para o tratamento de várias doenças (BROWN; POUDYAL; PANCHAL, 2015; SCOLARO; SOO JIN KIM; DE CASTRO, 2018).

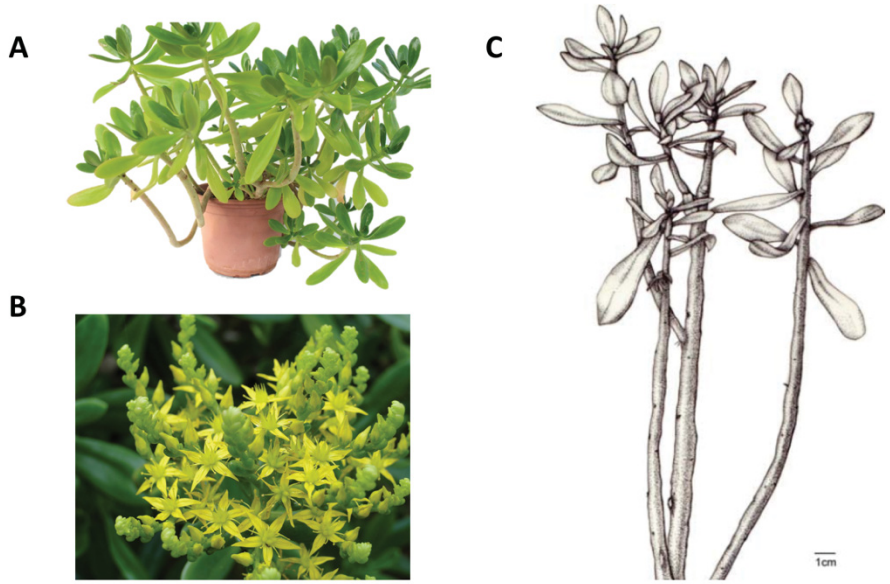
Considerando esta temática, o estudo de plantas e/ou alimentos funcionais com potencial terapêutico ainda é de extrema importância, seja para a validação do uso popular de plantas medicinais ou para contribuir para descoberta de novos fármacos.

1.2. *Sedum dendroideum*

Popularmente conhecido como Bálsamo, o *Sedum dendroideum* Moc *et Sessé* ex DC é uma planta suculenta de origem Sul Africana, amplamente distribuída pelo Brasil, e utilizada na medicina tradicional popular em diversos locais. Suas folhas, consumidas na forma de infusão, suco ou salada, possuem indicações populares para o tratamento de distúrbios gástricos, inflamações, diabetes e como agente contraceptivo (LIPORACCI; SIMÃO, 2013; ANDRADE-CETTO; HEINRICH, 2005; SILVA-TORRES *et al*., 2003; CARLINI, *et al*., 1970).

Pertencente a ordem Rosales, família Crassulaceae o gênero *Sedum* possui em torno de 18 espécies. Em relação a descrição botânica, o *S. dendroideum* é uma espécie subarbustiva, em média 1 metro de altura, suas folhas suculentas possuem formato obovada, com comprimento entre 1 a 5 cm e 1 a 2 cm de largura, sendo estas alternas e simples (DO ROCIO DUARTE; ZANETI, 2002) (Figura 1).

Figura 1. Sedum dendroideum Moc et Sessé ex DC (A). Inflorescência da espécie S. dendroideum (B). Ilustração cientifica do S. dendroideum (C).



FONTE: Figura adaptada de Do Rocio Duarte e Zaneti, 2002.

O gênero *Sedum* é conhecido pelos distintos grupos de compostos químicos, como flavonoides, com atividade pró-apoptótica em células hepáticas estreladas (LIN; LUO; JIN, 2015), alcalóides com efeito antiproliferativo em células tumorais (*Sedum sarmentosum*) (KANG *et al*., 2000) e polissacarídeos com atividade anti-inflamatória (SENDL *et al*., 1993).

De fato, estudos prévios confirmaram alguns efeitos biológicos de extratos e compostos isolados do *Sedum dendroideum*, tais como efeito analgésico (MALVAR *et al*., 2004; DE MELO *et al*., 2005), anti-úlcera (CARRASCO *et al*., 2014) e analgésico e anti-inflamatório do glicosídeo kaempferol (DE MELO *et al*., 2009) e antidiabético da kaempferitrina (SILVA *et al*., 2014).

1.3. DESORDENS DO TRATO GASTROINTESTINAL: ÚLCERAS GÁSTRICAS

O trato gastrintestinal é responsável pela digestão, absorção fornecendo ao organismo um suplemento constante de água e nutrientes necessário, além da excreção das substâncias ingeridas, sendo fundamental para manutenção da homeostase do organismo (HALL, 2017). As desordens do trato gastrointestinal afetam grande parte da população, sendo que a úlcera péptica vem se tornando uma das principais doenças,

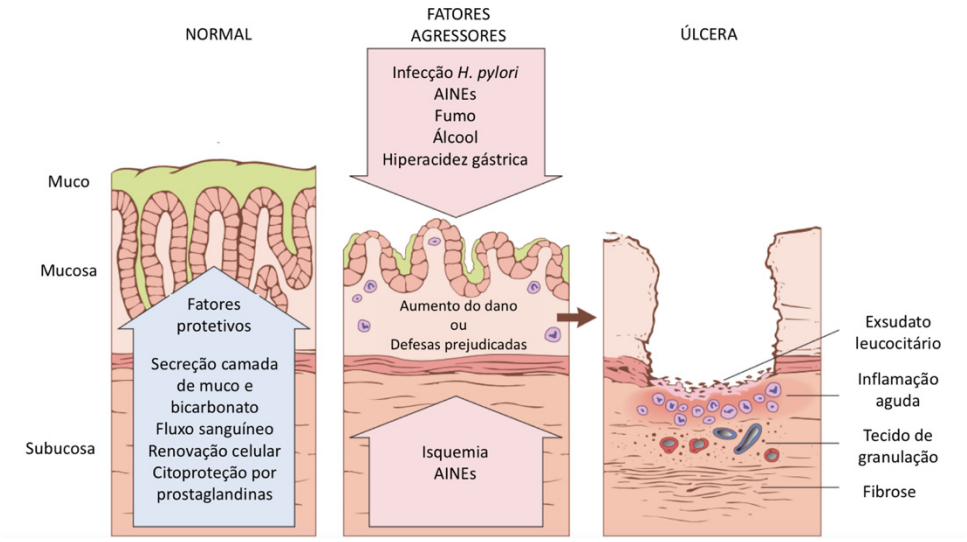
afetando cerca de 10% da população de todo o mundo (ZAKARIA *et al*., 2014). Aproximadamente 25 bilhões de americanos são afetados por úlcera gástricas, gerando um custo estimado de 9 bilhões de dólares (PATEL *et al*., 2012), e no Brasil dados demonstraram uma prevalência de 0,2 e 0,1% em homens e mulheres, respectivamente (OLIVEIRA *et al*., 2015).

São denominadas úlceras pépticas as lesões que ocorrem na região gástrica, duodenal e intestinal, como resultado do desequilíbrio entre os fatores de proteção e agressores da mucosa.

Os fatores agressores da mucosa podem ser endógenos, representados pelo excesso de secreção ácida e de pepsina, ou exógenos, como o consumo de álcool, anti-inflamatórios não-esteroides (AINEs) e fumo. Outro fator de relevância para formação de úlceras gástricas é a presença de infecção causada por *Helicobacter pylori* (MALFERTHEINER; CHAN; MCCOLL, 2009; LANAS; CHAN, 2017), bactéria gram negativa que utiliza hidrogênio disponível na mucosa gástrica como fonte de energia, descoberta que revolucionou a microbiologia gástrica e o tratamento da úlcera péptica, rendendo aos pesquisadores Dr. Barry Warren e John Marshal o prêmio Nobel de Fisiologia em 2005 (EUSEBI; ZAGARI; BAZZOLI, 2014).

Sabe-se que quando a mucosa gástrica é exposta a agentes agressores endógenos e exógenos, ocorre a destruição da camada muco-bicarbonato e consequentemente, a formação de lesão necrótica, geração de radicais livres e diminuição da oferta de oxigênio e nutrientes, resultando em uma reação inflamatória local. Durante este processo, ocorre migração de neutrófilos e macrófagos, que por sua vez irão fagocitar o tecido gástrico que foi danificado, liberando citocinas pró-inflamatórias, incluindo o TNF-α, IL-1α e IL-1β. Ainda, ocorrem alterações na microcirculação da mucosa gástrica, formação de edema, espécies reativas de oxigênio (ROS) e finalmente, a formação da úlcera péptica (ARAKAWA *et al.*, 2012; TARNAWSKI; AHLUWALIA; K JONES, 2013) (Figura 2).

Figura 2. Fatores protetores e agressores da mucosa gástrica e seu impacto na formação de úlceras gástricas.



FONTE: Adaptado: KUMAR et al., 2005.

Em relação aos fatores de proteção, o organismo possui defesas contra a alta concentração de ácido no lúmen do estômago e também no esôfago, como a produção de muco, que auxilia na proteção das células epiteliais impedindo a ação de moléculas ácidas e pepsina. A produção de muco, primeira linha de defesa do trato gastrointestinal, é estimulada pelas prostaglandinas E_2 e I_2, que também inibem diretamente a secreção de ácido gástrico pelas células parietais, sendo gastroprotetores endógenos (BRUNTON; CHABNER; KNOLLMANN, 2018). O muco é formado por um gel aderente com propriedades hidrofóbicas constituído de fosfolipídios surfactantes, água, glicoproteínas e bicarbonato, este último secretado pela superfície epitelial para manter o pH do microambiente em torno de 7, prevenindo a penetração de pepsina e digestão proteolítica (LAINE; TAKEUCHI; TARNAWSKI, 2008).

Outro mecanismo de defesa do estômago para proteção é a renovação celular constante. As células da mucosa gástrica têm uma taxa de renovação celular que varia de acordo com a demanda necessária para manter a saúde do tecido durante processos inflamatórios, como ocorre durante o processo ulcerogênico (MODLIN *et al*., 2003). A renovação celular é controlada por fatores de crescimento, hormônios e outros sinalizadores químicos, sendo importante destacar o fator de crescimento transformador alfa (TGF-α), fator de crescimento semelhante à insulina tipo 1 (IGF-1), prostaglandinas e gastrina (MODLIN *et al.*, 2003; LAINE; TAKEUCHI; TARNAWSKI, 2008). Em adição, o óxido nítrico (NO) também tem função citoprotetora, e como um potente

vasodilatador tem papel chave na microcirculação do estômago contribuindo para o aporte de oxigênio e remoção de substâncias nocivas (EL-DEMERDASH *et al*., 2010). O ácido gástrico, através da estimulação de receptores TRPV1 presentes em nervos sensoriais na mucosa, promove a ativação da enzima oxido nítrico sintase endotelial (eNOS), levando ao aumento na síntese de NO, que por sua vez estimula a produção muco, inibe a secreção gástrica através da supressão da liberação de histamina e mantém o fluxo sanguíneo e a integridade vascular (KATO *et al.*, 1998; HAN *et al.*, 2017).

Ainda, o sistema antioxidante enzimático e não-enzimático também tem grande importância na proteção do tecido gástrico. As ROS são geradas normalmente durante o processo de respiração mitocondrial como mecanismo de defesa do organismo contra patógenos, contudo fatores exógenos, como por exemplo, o uso de AINEs podem contribuir para o aumento excessivo da produção dessas espécies, levando ao estresse oxidativo, que pode gerar danos a componentes celulares como lipídeos, proteínas e DNA (KUMAR *et al*., 2005). Os sistemas antioxidantes enzimáticos e não enzimáticos estão presentes na mucosa do estômago para evitar a formação de lesões. Os principais sistemas enzimáticos contra estresse oxidativo são as enzimas superóxido dismutase (SOD) que catalisam a dismutação do O_2^- em O_2 + H_2O_2, para que então um segundo sistema enzimático, representado pela catalase (CAT), promova a quebra do H_2O_2 formado pela SOD em água. Juntas, essas enzimas providenciam a principal defesa enzimática contra as ROS no organismo. Em relação ao sistema antioxidante não-enzimático, podemos citar a glutationa (GSH), um tiol sulfidrílico não-protéico sintetizado a partir da cisteína absorvida da alimentação. O GSH é o principal antioxidante de organismos eucariotos, servindo com barreira antioxante e tem papel fundamental como cofator enzimático de duas enzimas do sistema antioxidante: glutationa peroxidase (GPx) que faz a redução de peróxidos e glutationa S-transferase (GST) que age na biotransformação e eliminação de xenobióticos (BHATTACHARYYA *et al.*, 2014).

O funcionamento de todos os fatores de proteção da mucosa gástrica é importante para evitar a formação de úlceras gástricas, contudo a presença desses fatores durante a cicatrização de lesões gástricas também é de grande importância. A cicatrização de úlceras gástricas envolve vários processos complexos que são controlados por fatores de crescimento, hormônios, citocinas inflamatórias e outros fatores de transcrição (TARNAWSKI; AHLUWALIA, 2012). A cicatrização das úlceras gástricas acontece através de um processo complexo que requer regeneração tecidual. É necessário que ocorra a síntese de fatores de crescimento, como por exemplo, fator de crescimento

epidérmico (EGF) e fator de crescimento endotelial vascular (VEGF), seguido de proliferação de novas células e migração das mesmas, re-epitelização, reconstrução glandular, formação de novos vasos sanguíneos para a restauração do sistema microvascular, e finalmente formação de tecido conectivo e da lâmina própria para a reparação tecidual total (TARNAWSKI; AHLUWALIA, 2012).

1.4 FARMACOTERAPIA DA ÚLCERA PÉPTICA

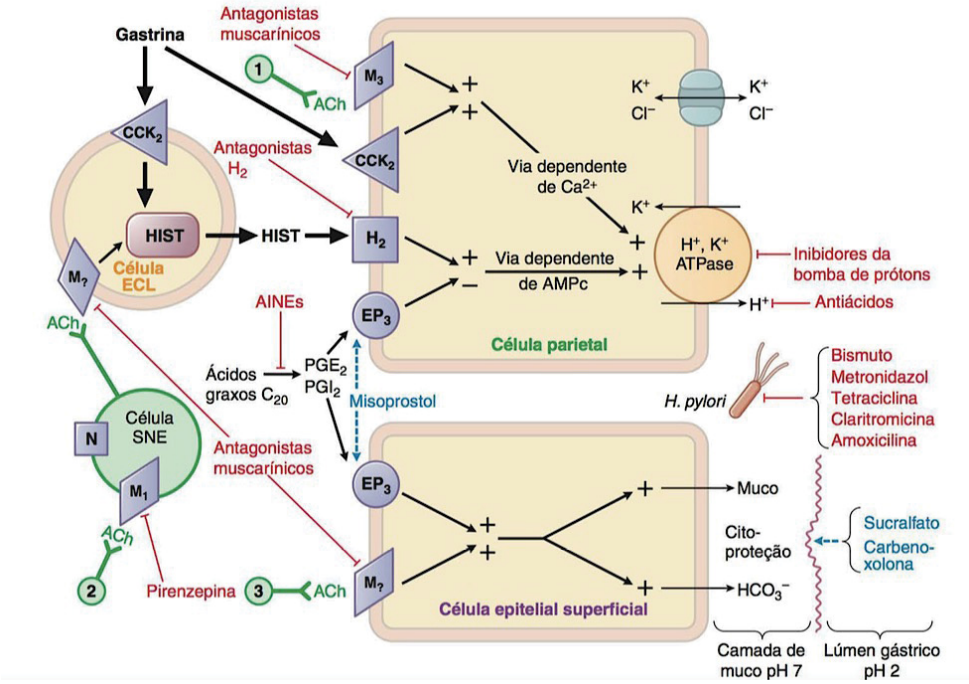
Atualmente os tratamentos disponíveis para úlcera gástrica ou distúrbios ácido-pépticos, agem principalmente na inibição/modulação da secreção gástrica e proteção do tecido durante o processo de cicatrização das lesões (BRUNTON; CHABNER; KNOLLMANN, 2018; MALFERTHEINER; CHAN; MCCOLL, 2009) (Figura 3). Existem cinco classes de fármacos usualmente utilizados para o tratamento de úlceras pépticas:

- Antiácidos, classe de medicamento que age pela neutralização do suco gástrico, diminuindo a exposição da mucosa gástrica a ácido gástrico e pepsina;
- Análogos de prostaglandinas, outra classe que tem seu mecanismo de ação baseado no aumento da resistência da mucosa, pela estimulação da produção de muco, secreção de bicarbonato e aumento do fluxo sanguíneo local.
- Agentes de barreira como sucralfato, composto formado por sacarose sulfatada e alumínio que se adere nas células epiteliais e no exsudato leucocitário da úlcera facilitando a cicatrização da lesão;
- Antagonista dos receptores H_2, agem competindo pela ligação ao receptor H_2 com a histamina, modulando a secreção ácida no estômago;
- Inibidores de bomba de prótons (H+/K+ APTase), classe de medicamentos mais utilizada, age inibindo irreversivelmente a secreção ácida através da inibição das bombas de prótons nos canalículos das células parietais.

A descoberta da infecção de *Helicobacter pylori* como agente agressor da mucosa e responsável pelo desenvolvimento de úlceras gástricas fez surgir uma nova recomendação de terapêutica tripla para tratamento de úlceras gástricas, composta de inibidores da secreção gástrica como os inibidores de bomba de prótons combinado com

dois antibióticos como metronidazol, tetraciclina, claritromicina ou amoxicilina (MALFERTHEINER; CHAN; MCCOLL, 2009).

Figura 3. Farmacoterapia de úlceras gástricas.



FONTE: BRUNTON; CHABNER; KNOLLMANN, 2018.

Entretanto, recentemente diversos trabalhos foram publicados relatando efeitos adversos relacionados a inibição da secreção ácida gástrica. Em uma revisão, Vaezi e colaboradores (2017) relataram os principais efeitos indesejados decorrentes do uso prolongado de inibidores de secreção gástrica: a diminuição da acidez estomacal diminui a absorção de diversos nutrientes que precisam de pH baixo para serem mais facilmente absorvidos como ferro, cálcio e vitamina B12, esses fatores estão diretamente ligados com o surgimento ao longo de prazo de demência, osteoporose e anemia, respectivamente. A alteração do pH também pode levar a proliferação de microrganismos aeróbicos no estômago que podem chegar no trato respiratório e estarem ligado a casos de pneumonia, a alteração do pH do trato gastrointestinal também afeta a composição normal da flora intestinal, aumentando as infecções por *Clostridium difficile*. Mais recentemente, Naito et al. (2018) reforçaram, com o uso de meta-analises, que a disbiose causada pela diminuição da acidez do trato digestório em curtos prazos também favorece a infecção por bactérias prejudiciais à saúde.

Em razão dos diversos efeitos adversos exibidos pela terapia de inibição da secreção gástrica, a busca por novas alternativas para o tratamento de úlceras péptica com um menor número de efeitos adversos e de fácil acesso a população se torna extremamente importante. Deste modo, as pesquisas têm como objetivo usufruir dos benefícios de produtos naturais com atividade gastroprotetora, que podem proporcionar além de um baixo custo de produção, também uma menor incidência de efeitos colaterais quando comparado a síntese de novos compostos sintéticos (KRAFT; LANGHORST, 2014).

1.5 FITOTERAPIA PARA DESORDENS DO TRATO GASTRINTESTINAL

A medicina tradicional utiliza diversas plantas para o tratamento de doenças gastrointestinais e recentemente muitos esforços estão sendo feitos para identificação de princípios ativos destes compostos (PATEL *et al*, 2012).

Segundo a Organização Mundial da Saúde, as plantas são o primeiro elemento de cuidado da saúde para grande parte da população mundial (TOMLINSON; OLAYIWOLA, 1998). Hoje a maioria das farmacopeias do mundo prescrevem medicamentos obtidos a partir de extratos de plantas, sendo a Alemanha o maior produtor e consumidor europeu, onde a fitoterapia é altamente empregada, baseada na eficiência da dose e compostos ativos identificados (PETROVSKA, 2012).

Fitoterápicos são medicamentos desenvolvidos exclusivamente de matérias-primas vegetais que possuem alguma ação farmacológica ativa. Nesse ponto o Brasil ganha um grande destaque, devido a seu extenso território chega a abranger cerca de 15 a 20% de biodiversidade de plantas superiores (VARELA; AZEVEDO, 2013). O interesse institucional pelo uso de plantas medicinais iniciou na década de 80, com a resolução Ciplan nº 8/1988, que deu início a regulamentação da fitoterapia nos serviços básicos de saúde e assistência médica.

Em 1996, durante a 10ª Conferência Nacional de Saúde foi redigido um relatório incentivando a incorporação no sistema único de saúde (SUS) de práticas de terapias alternativas e práticas populares tradicionais como a fitoterapia, acupuntura e homeopatia. Ainda neste relatório, foi apontado que o Ministério da Saúde deveria incentivar a fitoterapia e elaborar normas para sua utilização. (BRASIL, 2015a).

Em 2006 foi instituído pelo Governo Federal por meio de decreto nº 5.813 o Programa Nacional de Plantas Medicinais e Fitoterápicos que tem como objetivo garantir

o uso seguro e racional de plantas medicinais e fitoterápicos. O decreto visa também promover o uso sustentável de plantas medicinais tradicionais, estabelecendo fomento para pesquisas e inovação em fitoterápicos aliado ao desenvolvimento sustentável da biodiversidade (BRASIL, 2006b). No mesmo ano o Ministério da Saúde implementou a Política Nacional de Práticas Integrativas e Complementares no SUS por meio da portaria nº 971/2006 que tem por objetivo incorporar de maneira permanente práticas de terapias alternativas antes já incentivadas pelo Ministério da Saúde.

Os avanços para o uso de fitoterapia no sistema único de saúde continuaram, em 2011 o Ministério da Saúde aprovou uma nova resolução, RDC Nº 60 de novembro de 2011 que promove o Formulário de Fitoterápicos da Farmacopeia Brasileira, contendo informações de medicamentos fitoterápicos como a forma correta de preparo, as indicações e as restrições de cada planta medicinal citada nessa farmacopeia. Em 2013 foi implementada por meio da RDC nº 13 de março de 2013 as Boas Práticas de Fabricação de Medicamentos Fitoterápicos Tradicionais, norma que foi estabelecida para normatizar a fabricação de produtos fitoterápicos tradicionais garantindo a qualidade necessário para o uso desses produtos.

Outra inovação na regulamentação dos fitoterápicos foi a RDC nº 26/2014 adicionou um novo conceito para os produtos derivados de plantas medicinais, Produto Tradicional Fitoterápico (PTF), com o objeto de facilitar o registro de fitoterápicos pois diferente de medicamentos fitoterápicos, os PTF têm sua eficácia assegurada pelo conhecimento etnofarmacológico e dados descritos na literatura, sendo dessa forma isentos de testes clínicos exigidos para medicamentos sintéticos e fitoterápicos usuais.

Com potencial já reconhecido, medicamentos fitoterápicos encontram-se incluídos na Relação Nacional de Medicamentos Essenciais (RENAMA) e são fornecidos pelas unidades básicas de saúde quando receitado por um profissional da saúde. Para o tratamento de úlceras gástricas, gastrite e outras desordens do trato gastrointestinal estão disponíveis alguns fitoterápicos como por exemplo: *Maytenus ilicifolia,* popularmente conhecida como Espinheira-santa; *Schinus terebinthifolius,* popularmente conhecida como Aroeira; *Rhamnus purshiana*, conhecida popularmente como Cáscara-sagrada, que apresenta atividade pró-cinética intestinal; e *Mentha piperita*, conhecida como hortelã, amplamente utilizada e com indicações para tratamento da síndrome do intestino irritável.

Levando em consideração que o Brasil aponta como maior produtor de pesquisa com produtos naturais na América Latina, seria esperado a produção de inúmeros fitoterápicos pelo país. Entretanto, grande parte dessa pesquisa é realiza em universidades

e não chega efetivamente a população na forma de fitoterápicos. Devido a isso nos últimos anos houve um aumento no número de parcerias entre universidades e indústrias farmacêuticas nacionais com o objetivo de desenvolver medicamentos baseados nos achados acadêmicos (CALIXTO; SIQUEIRA JUNIOR, 2008; DUTRA *et al*., 2016).

Um importante e bem sucedido exemplo da parceria entre academia e indústria farmacêutica foi o desenvolvimento do primeiro medicamento fitoterápico aprovado no Brasil para tratamento de gastrite leve a moderada. O medicamento Kios®, que tem como princípio-ativo a *Schinus terebinthifolius* (aroeira), uma planta nativa da América do Sul, foi produzido pela empresa farmacêutica Hebron em parceria com as Universidades Federal do Rio Grande do Norte (UFRN) e de Pernambuco (UFPE) (MEDEIROS et al., 2007).

1.6 OBJETIVOS

1.6.1 Objetivo geral

Investigar o efeito gastroprotetor, cicatrizante gástrico e pró-cinético do infuso preparado com as folhas de *Sedum dendroideum* (SDI), assim como o mecanismo de ação envolvido nas atividades biológicas estudadas.

1.6.2 Objetivos específicos

- Caracterizar fitoquímicamente o extrato aquoso preparado pela infusão das folhas de *Sedum dendroideum* (SDI) e quantificar metabólitos secundários;

- Avaliar o efeito gastroprotetor da SDI no modelo de lesões agudas induzidas por etanol em ratos, investigando a participação de fatores protetores como muco, GSH e microcirculação;

- Avaliar o efeito gastroprotetor da SDI no modelo de lesões agudas induzidas por indometacina em ratos; investigando a participação de fatores protetores como muco e GSH;

- Avaliar o efeito anti-secretor gástrico da SDI no modelo de ligadura do piloro em ratos;

- Avaliar o efeito da SDI sobre a motilidade gastrointestinal em camundongos;

- Avaliar a ação cicatrizante da SDI no modelo de úlcera crônica induzida por ácido acético em ratos, investigando a preservação de muco, a proliferação celular, parâmetros inflamatórios e de estresse oxidativo, bem como parâmetros de toxicidade.

3. ARTIGO CIENTÍFICO 1

Artigo cientifico submetido para publicação na revista Journal of Ethnopharmacology

Manuscript Draft

Title: Chemical composition, antioxidant and gastrointestinal properties of Sedum dendroideum Moc & Sessé ex DC leaves tea infusion

Article Type: Research Article (max 7,500 words)

Keywords: LC-MS/MS; chemical constituents; phenolic compounds; flavonoids; antioxidant; cytotoxic effects; antiulcer activity; gastrointestinal motility.

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Abstract: Sedum dendroideum is an edible plant employed in Brazilian folk medicine for the treatment of gastric disorders. The purpose of this study was to elucidate the chemical constituents, antioxidant, cytotoxic and gastrointestinal properties of Sedum dendroideum infusion (SDI) leaves. Phytochemical analysis revealed the presence of different flavonol glycosides, containing myricetin and quercetin, along with the kaempferol as aglycones. In vitro pharmacological investigation of SDI demonstrated potent antioxidant activity in DPPH assay and absence of cytotoxicity in Caco-2 cells by MTT method. SDI promoted gastroprotection against ethanol or indomethacin through reinforcement of gastric wall mucus, GSH content and nitric oxide release, without present antisecretory properties. Furthermore, SDI increase small bowel transit through cholinergic pathways. In conclusion, SDI features a chemical profile that contributes to these gastric health-promoting effects.

3.1 Chemical composition, antioxidant and gastrointestinal properties of Sedum dendroideum Moc & Sessé ex DC leaves tea infusion

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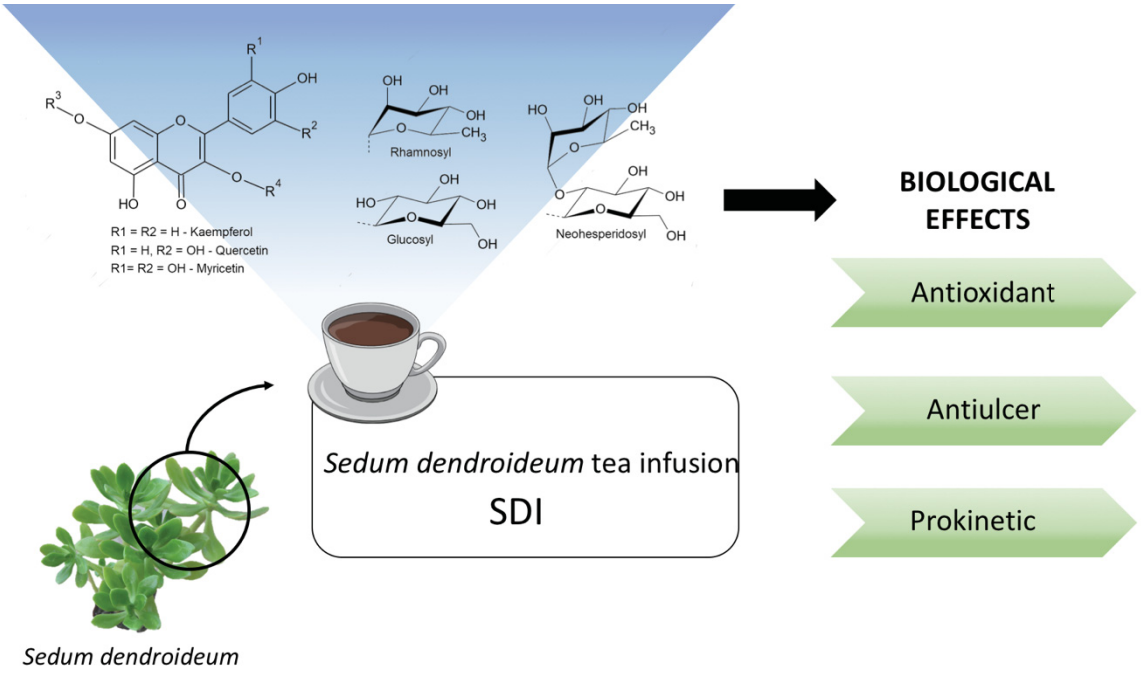
Declarations of interest: none

Graphical abstract



1. Introduction

Sedum dendroideum Moc. & Sessé ex DC. (Crassulaceae) is a medicinal plant employed in Mexican and Brazilian culture for treatment of diabetes (Andrade-Cetto & Heinrich, 2005), eye inflammation and as contraceptive agent (Silva-Torres et al., 2003). In Brazil is popularly known as "bálsamo", and popular reports described the use in form of juice or infusion prepared by soaking the leaves in hot water for treatment for the treatment of gastric ulcer (Carlini, Neto, Almeida, & Marigo, 1970; Rosas-Piñón *et al*., 2012).

Many bioactivities of extracts and compounds isolated from *Sedum dendroideum* have been researched. With an incontestable pharmacological effect, previous studies showed that the *Sedum dendroideum* leaf juice exhibited antinociceptive and anti-inflammatory activities (De Melo *et al*., 2005), and when isolated glicosideos like kaempferol and kaempferitin promoted antinociceptive and anti-inflammatory effects in acetic acid-induced writhing and hypoglycemic activity in mice with streptozotocin-induced diabetes respectively (De Melo *et al.,* 2009; Da Silva *et al.*, 2014). Extracts also showed biological effect, as demonstrated by Carrasco and coworkers (2014) with an hydroethanolic extract containing flavonoids, phenols, and tannins that presented gastroprotective action in rats. However, the population does not consume alcoholic extracts as mentioned above with therapeutic purposes, being necessary also the validation of the popular use sedum dendroideum infusion or juice in gastrointestinal disorders.

The adequate gastric function is essential for digestion and absorption of nutrients. However, disorders of the gastrointestinal tract as peptic ulcers are common, causing discomfort and abdominal pain. Peptic ulcers occurs due to exposition to acid and pepsin associated with the decrease of protective mechanisms of the mucosa, such impairment

of mucus layer, antioxidant system and blood flux, which together to lifestyle habits, contributes to the gastric ulcer formation (Yandrapu & Sarosiek, 2015).

Considering that herbal medicines contain several bioactive metabolites, the aim of the present study was to characterize the chemical constituents and evaluate the antioxidant, antiulcer and prokinetic effects of an infusion prepared with leaves of *Sedum dendroideum* (SDI).

2. Materials and methods

2.1. Botanical material and infusion preparation

Sedum dendroideum was harvested in Campina Grande do Sul, PR, Brazil and identified by Dr. José Tadeu Weidlich Motta, plant taxonomist and curator of Municipal Botanical Museum (MBM) of Curitiba, PR, Brazil. A representative voucher specimen is deposited at the MBM herbarium (MBM-272917).

The infusion was prepared as previously published (De Oliveira *et al.*, 2018). Briefly, 1.25 kg of dried leaves of *Sedum dendroideum* were submitted to extraction with boiling water (100 g/L) by infusion during 1 h. SDI was lyophilized in order to obtain a dry extract to determine the infusion concentration for perform *in vitro* and *in vivo* assays.

2.2. Phytochemical analysis

2.2.1. Liquid chromatography-mass spectrometry of SDI

The chromatography analysis was carried out in a high-performance liquid chromatography (HPLC, Agilent 1200) coupled to a mass spectrometry (MS) detector. The analysis were developed in a reversed-phase chromatography employing a BEH C18

column (50 x 2.1 mm with particle of 1.7 µm, from Waters), using ultra-pure water (Milli-Q) and acetonitrile (J.T. Baker) containing 0.1% of formic acid (v/v). A gradient of acetonitrile was developed, increasing from 5 to 20 % (in 5 min), to 80% (in 10 min), returning to 5% (in 11 min), at 350 µL/min, with the column temperature at 60 ºC and pressure did not exceed 4500 psi. Between analyses, the column was balanced for 3 min with the initial solvent. The sample was dissolved in MeOH-H_2O (1:1, v/v at 2 mg/mL) and 10 µL was injected.

The mass spectrometry analysis was developed with an electrospray ionization in a LTQ-Orbitrap XL (Thermo Scientific) at atmospheric pressure ionization (API), in the positive and negative ionization modes. The sample from LC was dried by a flow of nitrogen in the sheath gas and auxiliary gas (at 40 and 5 arbitrary units, respectively), with the source temperature of 300 ºC. Positive and negative ions were obtained, however in the negative ionization mode, the compounds were better observed and fragmented. The negative ions were obtained using the spray at 3.2 kV, the tube lens at -200 V and the capillary at -46 V. The MS data was acquired in total ion current (TIC) and a data dependent event was used to fragment the base ion each peak, using a pulsed quantum dissociation activation (PQD) with normalized collision energy of 35.

2.2.2. Determination of the total phenolic and flavonoid contents in SDI

The total phenolic content was performed by spectroscopy using the colorimetric method described by Singleton and coworkers (1999). In 96-well plates, 20 µL of SDI (1 mg/mL) was mixed with 100 µL Folin-Ciocalteau phenol reagent. After 5 min, 80 µL of Na_2CO_3 (7.5%) was added and incubated for 120 min. Absorbance was measured at 760 nm using an automated microplate reader (Epoch™ Microplate Spectrophotometer-BioTek, Winooski, VT, USA). The total phenolic content was determined by

interpolation of the samples absorbance with the standard curve of gallic acid (standard phenolic compound). The results were expressed in g gallic acid/100 g of sample.

Total flavonoid content was determined using rutine as the standard flavonoid [of (Quettier *et al.*, 2000). Briefly, 400 μL of SDI (1 mg/mL) were mixed with equal volume of $AlCl_3.6H_2O$ methanol solution (2 % w/v). After 10 min, the absorption of the standards was measured at 430 nm as previously described. The total flavonoid content was determined by interpolation of the samples absorbance with the standard curve of rutine. The results were expressed in g rutin/100 g of sample. All analyzes were performed in triplicate.

2.3. Determination of radical scavenging activity by DPPH *in vitro* method

The DPPH (2,2′-diphenylpicryl hydrazyl) free-radical scavenging assay was used to estimate the antioxidant ability of SDI (Blois, 1958). Briefly, 225 μL of SDI (1, 10, 100 and 1000 μg/mL), ascorbic acid (AA¸ 50 μg/mL as positive control) or distilled water (as negative control) were mixed with 75 μL of methanolic DPPH solution (40 μg/mL) in a 96-well plate for 5 min. The absorbance was measured at 517 nm and values obtained were interpolated in a standard curve of DPPH (0-60 μM). Results were expressed as % of inhibition of DPPH.

2.4. Caco-2 cell culture and cytotoxicity assay

Human colon carcinoma cells line (Caco-2) were purchased from the Cell Bank of Rio de Janeiro, Brazil. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) and Ham´s-F12 (1:1), supplemented with 10% fetal bovine serum (FBS) and 100 IU/ mL penicillin/streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Caco-2 cells were cultured in 96-well plates, at a density

of 7 x 10^3 cells/well, and treated with increasing concentrations (10, 100, and 1000 μg/mL) of SDI diluted in FBS free medium. After 24 h of incubation, the solution was removed and 100 μL of MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (0.5 mg/mL) was added to each well and incubated for 3 h at 37 °C. Then, MTT solution was aspired and 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Cell survival was assessed through absorbance determination at 570 nm. Medium alone was used as control and the cell viability was expressed as % of control cells.

2.5. Animals

Adult female Wistar rats (180-200 g) and *Swiss* mice (25-30 g) were provided by the Biotery of Federal University of Parana. Animals were housed under standard laboratory conditions: plastic cages (maximum of 5 rats and 20 mice per cage) with wood shaving bedding and free access to water and food, under a 12 h light/dark cycle and at controlled temperature (22 ± 2 ºC). Fasting (16 h) was used prior all assays. The animals were kept in cages with raised, wide-mesh floors to prevent coprophagy with free access to water. All experimental procedures and animal handling were conducted in agreement with the "Guide for the Care and Use of Laboratory Animals" (8^{th} edition, National Research Council, 2011) and approved by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO − UFPR 1010).

2.5.1. Dose conversion of SDI between human and animals

Popularly, *Sedum dendroideum* is consumed as infusion for treatment of gastric ulcers (prepared approximately with 5 leaves in 500 mL of hot water), three times a day.

Using the total weight of 5 dried leaves (~ 0.6 g) and the daily consumption, the dose was extrapolated to the ingestion of 12 mg/kg/day for humans with a mean weight of 70 kg (Leite, 14 Benefícios do Bálsamo). Then, based on allometric scaling approach, the normalization of the human dose (12 mg/kg) was performed according to body surface area, providing the equivalent doses of 80, 160 and 320 mg/kg for rats and 150, 300 and 600 mg/kg for mice (Nair & Jacob, 2016). For reducing the number of rats in each experimental group, the median effective dose (ED_{50}) values were determined based on inhibition of gastric lesion induced by ethanol following SDI oral administration. Thus, the dose of 191 mg/kg was chosen to evaluate the following gastroprotective activity of SDI.

2.5. Pharmacological analysis

2.5.1. Acute gastric lesions induced by ethanol

The acute hemorrhagic lesions were induced by oral administration (v.o.) of ethanol (Robert, Nezamis, Lancaster, & Hanchar, 1979). Animals were orally pretreated with water (Vehicle [V]: 1 mL/kg), omeprazole (O: 40 mg/kg) or SDI (80, 160 and 320 mg/kg, SDI ED_{50} 191 mg/kg, or SDI 19.1 mg/kg by intraperitoneal route, i.p.). Sixty or 30 min after oral or intraperitoneal treatments, respectively, all animals received ethanol P.A. (1 mL/rat) and then, animals were euthanized 1 h later by thiopental overdose (100 mg/kg, i.p.). To analyze the gastric lesions, the stomachs were excised, opened along the smaller curvature and photographed to provide visual evidence of hemorrhagic ulcers. All ulcer wound was measured by computerized planimetry using the program Image Tool® 3.0, and the lesion area was expressed in mm^2.

2.5.2. Involvement of endogenous nitric oxide in the gastroprotective effect of SDI

To investigate the involvement of endogenous nitric oxide (NO) in the gastroprotective effect of SDI, rats were pretreated with Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 20 mg/kg, i.p.). Following 30 min, the rats received a single oral dose of L-Arginine (L-Arg: 200 mg/kg) or SDI (ED_{50} 191mg/kg), 1 h before oral administration of ethanol P.A. (1 mL/rat). Then, animals were euthanized 1 h later, and gastric lesions were analyzed as described above.

2.5.3. Acute lesions induced by indomethacin

The rats were pretreated by oral route with water (vehicle [V]: 1 mL/kg), Prostaglandin E_2 (PGE_2: 20 μg/kg) and SDI (ED_{50} 191 mg/kg). One h after the treatments, all animals received a single oral dose of indomethacin (100 mg/kg) and then, animals were euthanized 6 h as previously described. The same procedure was employed to analyze the indomethacin-induced gastric lesions.

2.5.4. Determination of gastric wall mucus

The evaluation of gastric wall mucus content was performed in stomachs from ethanol- and indomethacin-induced lesions, according to the reported method (Corne, Morrissey, & Woods, 1974). First, the glandular segment of stomach was complexed with a dye solution of 0.1 % Alcian Blue during 2 h. After, the tissue was washed with 250 mM sucrose twice for 15 and 45 min respectively, and then the complex mucus-dye was extracted adding 500 mM magnesium chloride and stirred intermittently for 2 h. The solution extracted was mixed with the same ether volume and centrifuged for 10 min at

3600 rpm. The aqueous layer was separated to measure the absorbance at 580 nm and the results were expressed in μg Alcian blue/g of glandular tissue.

2.5.5. Determination of gastric glutathione levels

The samples of stomach from ethanol- and indomethacin-induced lesions were homogenized with cold 200 mM potassium phosphate buffer (pH 6.5) in a volume equal to 3 times the weight of fresh gastric tissue to determinate glutathione (GSH) levels as previously described (Sedlak & Lindsay, 1968). Aliquots of samples were mixed and vigorously shaken with 12.5% trichloroacetic acid (ATC) before being centrifuged at 3000 rpm for 15 min at 4 ºC. The supernatant of the samples, 400 mM TRIS-HCl buffer (pH 8.5) and 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added in a 96-well plate to perform the colorimetric reaction. Then, the absorbance was read at 412 nm. The values obtained were interpolated in a standard curve of GSH and the results were expressed as μg GSH/g of tissue.

2.5.6. Induction of hypersecretion by pylorus ligation in rats

The gastric secretion was evaluated as previously described (Shay, 1945). The rats were anesthetized with halothane, and after laparotomy, the stomach was exposed and the pylorus was tied. The animals were treated through intraduodenal route with vehicle (V: water 1 mL/kg, i.d.), SDI (ED_{50} 191 mg/kg, i.d.) or by oral route with the positive control omeprazole (O: 40 mg/kg, v.o.), 1 h before the pylorus ligation. After 4 h, animals were euthanized, the stomach was pinched, and gastric juice collected and centrifuged to measurements of the secreted volume, pH and total acidity.

2.5.7. Evaluation of prokinetic properties through gastric emptying and intestinal motility

For determinate the gastric emptying and small intestinal transit, mice were pretreated by oral route with vehicle (V: Water 1 mL/kg) and SDI (150, 300 and 600 mg/kg). After 1 h, all animals received 1.5% phenol red marker (0.5 mL/mice) and then, animals were euthanized 20 min later. The abdominal cavity was opened and the stomach and the small intestine until the cecum were immediately removed for evaluation of phenol red marker position (Suchitra, 1979).

To measure the gastric emptying, the stomach and its contents were homogenized with water and centrifuged for 15 min at 1500 rpm. Then, 150 μL of the supernatant plus 150 μL of 0.1 N NaOH were added in a 96-well plate for colorimetric reaction and reading at 560 nm. Gastric emptying was measured as the amount of marker that remained in the stomach at the end of the experiment, and the results were expressed as %.

In another set of experiments, to address some of the mechanisms by which SDI modulates small intestinal transit, mice were pretreated subcutaneously with atropine (5 mg/kg, s.c.) or orally with loperamide (5 mg/kg, v.o.), 30 or 60 min, respectively, before the treatment with SDI (150 mg/kg, v.o.). Following 1 h, all animals received 1.5% phenol red marker (0.5 mL/mice). Then, the small intestine was dissected from the pylorus to the ileocaecal junction and the intestinal transit was measured considering the total length of the small intestine and the distance covered by phenol red solution. The results were expressed as %.

2.6. Statistical analysis

Data were expressed as mean ± standard error of the means (S.E.M.). Statistical differences between experimental groups (n= 6-9 animals per group) were analyzed with

one-way ANOVA followed by Bonferroni's multi-comparison post-hoc test. The ED_{50} values (effective dose capable of inhibiting the gastric lesions formation by 50% relative to the control group values) or IC_{50} values (inhibitory concentration required to obtain a 50% antioxidant effect in DPPH assay) were determined by nonlinear regression analysis and reported as geometric mean. All analyzes were performed using the GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA). Differences were significant when $P \leq 0.05$.

3. Results and discussion

3.1. Phytochemical investigation

In previous investigation (De Melo, et al., 2005; De Melo, et al., 2009; Da Silva, et al., 2014), some flavonol glycosides from *Sedum dendroideum* were identified, containing mainly kaempferol as aglycone. De Melo and coworkers (2009) founded the kaempferol attached mainly by rhamnose (Rha), with glucose (Glc) at lower abundance, linked in the positions 3 and/or 7.

In our current analysis, using LC-MS in the negative ionization to produce depronated ions $[M-H]^-$, we have found different flavonol glycosides, containing myricetin and quercetin, along with the kaempferol as aglycones (Figure 1A). In SDI, the first peak (**1**) was observed at m/z 479.2 with fragments at *m/z* 317.0 and 316.0, being consistent with myricetin-hexoside. The fragments observed are characteristics of myricetin, produced two types of linkage breakdown, the heterolytic cleavage, yielding the regular ion (i.e. *m/z* 317.0) and, by a homolytic cleavage, a radical ion was produced (i.e. *m/z* $316.0^{\cdot}$). Similarly, the other aglycones also produced these two ions from aglycones, being at *m/z* 285/284 for kaempferol and *m/z* 301/300 for quercetin. However,

since these flavonols can be linked to glycans at two positions, each linkage could undergoes to a hetero- and/or hemolytic cleavage, yielding to different ions. The superscript symbol (·) will be used to indicate a radical ion.

The peak **2**, at *m/z* 739.3 with main fragments at *m/z* 593.3 (-Rha), 447.2 (-Rha_2), 430.1· (-Rha, -Glc) and 284.0· (from kaempferol), being consistent with kaempferol 3-*O*-neohesperidoside 7-*O*-rhamnoside (Melo, et al., 2005; De Melo, et al., 2009; Da Silva, et al., 2014). The peak **3**, at m/z 609.3 and fragments at *m/z* 462.1·, 317.0 and 315.1·· was consistent with myricetin 3-*O*-rhamnoside 7-*O*-rhamnoside. The ion at *m/z* 315.1·· was assigned as a product from a double homolytic cleavage, indicative of two glycosylation sites. The peak **4**, at *m/z* 609.3 had different fragments from peak **3**, with those at *m/z* 463.2, 447.2/446.3· and the aglycone ions at *m/z* 301.1, 300.1· and 299.0··, suggesting a quercetin 3-*O*-glucoside 7-*O*-rhamnoside.

The abundant peak **5**, was found at *m/z* 593.3 and fragments at *m/z* 447.2, 431.2/430.2· and the aglycone at *m/z* 285.0 (main), 284.0· and 283.0·· (lower). This compound is consistent with the kaempferol 3-*O*-glucoside 7-*O*-rhamnoside. The most abundant peak (**6**) was consistent with kaempferitrin (kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside), observed at *m/z* 577.2 and fragments at *m/z* 431.1/430.1· and 285.0/283.1··. Another abundant peak (**7**) gave the ion at *m/z* 737.3 and main fragments at *m/z* 675.3, 635.3, 593.2, 429.2 and 284.0·. Although the fragments at m/z 284.0· and 593.3 could suggest a kaempferol-gluco-rhamnoside, the other fragments could not be property identified. The fragment at *m/z* 635.3 is consistent with an acetyl group linked to glycoside, however the fragment at *m/z* 675.3 was not assigned. Porter et al (2012) have characterized some flavonol glycosides with a substituent of 3-hydroxy-3-mehtylglucaric acid. Although the structure from Porter and coworkers had similar ion at *m/z* 737, with our fragmentation profile we could not confirm the identity of peak **7**.

The peak **8**, observed at *m/z* 577.3 and fragments at *m/z* 431.2 and 285.1 was consistent with an isomer of kaempferitrin. The peak **9** at *m/z* 693.3 and fragments at *m/z* 577.3, 431.1/430.2·, 285.0 was consistent with kaempferitrin containing an unknown substituent. The peak **10** appeared at *m/z* 591.3, and m/z 529.2, 489.2, 447.2 and 285.0/284.0·. The fragments at m/z 285.0/284.0· and 447.2 are consistent with a kaempferol glucoside, the ion at m/z 489.2 suggest an acetylation but the ion at *m/z* 529.2 was not identified. This compound has a similar substituent of peak **7**.

The peak **11** at *m/z* 431.2 and 285.0/284.0· is consistent with a kaempferol 3-*O*-rhamnoside, considering its lower abundance in relation to the isomer (peak **14**). The peak **12**, at *m/z* 539.4, gave rise to fragmentation profile different from common flavonoid-glycosides. However, the more intense product-ions at *m/z* 437.1 and 275.0 (neutral loss 162 atomic mass units) are consistent with glycoside. The peak **13** was not identified, observed at *m/z* 395.2, it had similar ions to peak **12**, with the product-ion at *m/z* 275.0 being the highest. The peak **14**, at m/z 431 and fragments at *m/z* 285/284.0· is consistent with kaempferol 7-*O*-rhamnoside, considering that kaempferol 7-*O*-glycosides are the main compounds in *Sedum dendroideum* (Melo, et al., 2005; De Melo, et al., 2009; Da Silva, et al., 2014). The peak **15**, was observed at *m/z* 793.4, with prominent fragments at *m/z* 635.2, 593.2 and 285.0/284.0·. The product-ions at *m/z* 285.0/284.0· are consistent with kaempferol and that at *m/z* 593.2, with a diglycoside (e.g. glucose + rhamnose). Similarly to the peak **7**, the fragment at *m/z* 635.2 is consistent with an acetyl group attached to kaempferol diglycoside, as previously observed (Abdel-Hameed, Bazaid , & Salman, 2013), however, an unknown group seems to be attached to this structure. The last peaks found in the chromatogram (**16**, **17**, **18**) were not identified (Figure 1B). The results are summarized in the Table 1.

The quantification of total phenolic compounds and flavonoid contents confirmed the presence of these antioxidant compounds in the *Sedum dendroideum* tea infusion (2.30 ± 0.21 and 1.12 ± 0.06 g/ 100 g SDI, respectively), which suggested that SDI contains considerable amount of important secondary metabolites (Figure 1C). Particularly, phenolic compounds are among the major phytochemical compounds responsible for the antioxidant activity of plants and dietary supplements (Guldiken *et al.*, 2018).

In fact, we observed that SDI had direct DPPH radical-scavenging ability. The scavenging effect of SDI on DPPH radicals was found to increase in concentration dependent manner (10, 100 and 1000 μg/mL), decreasing the DPPH free radicals in 37.74, 86.45 and 94.69% respectively, when compared to vehicle group (V: 8.52 ± 0.584 μM) (SDI IC_{50}: 13.25 ± 3.37 μg/mL). In addition, the ascorbic acid or vitamin C used as a positive control, an essential cofactor for several enzymes and powerful antioxidant, decreased the DPPH free radicals in 85.08% when compared to the vehicle group (Figure 2A). These results were expected because of the high percentage of phenolic components and flavonoids in SDI, and reinforce the notion that in tea infusions, these antioxidants components exert a positively correlation in terms of concentration (IC_{50}) (Fotakis et al., 2016). As shown in Figure 2B, the results from MTT assay demonstrate that 24 h incubation of Caco-2 cells with SDI 10-1000 μg/mL did not show changed cell viability when compared to control medium, demonstrating that SDI did not display cytotoxic effects.

3.2. Pharmacological investigation

Our results demonstrated that *Sedum dendroideum* infusion prepared with the leaves in according to the prepare traditional and the popular use of plant exert beneficial effects on gastrointestinal tract as an antiulcer and prokinetic agent.

It is well know that ethanol is an exogenous aggressive factor for ulcer development, causing directly gastric damage, leading to the formation of acute hemorrhagic lesions, as can be seen in the Figure 3D. Moreover, the destruction of protective factors such as mucus barrier and the depletion of GSH, also contribute to the development of the ethanol induced-ulcer, favoring the generation of reactive oxygen species (ROS), free radicals and lipoperoxidation, culminating in oxidative stress and further gastric injury (Yang et al., 2017). SDI administered by oral route at doses of 80, 160 and 320 mg/kg significantly reduced the ethanol-induced gastric lesions in 37.04, 44.06 and 63.04% respectively when compared to the vehicle control group (V: 198.93 ± 22.41 mm^2), with an ED_{50} of 191.00 ± 0.08 mg/kg. Omeprazole (40 mg/kg,) also prevented the ulcer formation in 93.35% (Figure 3A, 3D-H). The mucus-bicarbonate barrier constitutes the first line of defense of the gastric mucosa against acid and pepsin, maintaining luminal pH between 7.0. Accordingly, SDI (80, 160 and 320 mg/kg) preserved significantly the gastric mucus levels when compared to the vehicle control group in 36.84, 39.67 and 37.71%, respectively (V: 1899.15 ± 121.68 µg of Alcian blue/g of tissue), while omeprazole preserved mucus in 44.69% (Figure 3B). Moreover, the decrease of GSH levels, which is an essential endogenous antioxidant, greatly impairs its important action to limit the toxicity of ethanol (Brown, Harris, & Gauthier, 2004). As illustrated in Figure 3C, SDI (320 mg/kg) and omeprazole replenish the GSH levels in 63.35 and 61.88%, respectively, when compared to the vehicle control group (V: 788.57 ± 69.02 µg GSH/g of tissue).

In a subsequent experiment, rats were pretreated with SDI at 19.1 mg/kg, a 10-fold lower dose (in relation to the ED_{50} obtained with the oral administration of SDI), by intraperitoneal route to discard a possible physical barrier formation on the gastric mucosa prior to the ethanol administration. It is important to mention that only the ED_{50} was employed, aiming to reduce the number of experimental animals, in agreement to the 3Rs principles. Interestingly, the gastroprotective effect promoted by SDI remained. The intraperitoneal administration of SDI (19.1 mg/kg) significantly reduced the gastric lesions induced by ethanol in 86.25% and preserved the mucus and GSH depletion in 34.74 and 56.95%, respectively (Figure 4). These observations reinforce the notion that the gastroprotection promoted by oral administration of SDI does not occur only by a physical barrier formation, as occurs with sucralfate treatment, in which the main mechanism of action is attributable to the formation of a protective barrier over the eroded mucosa (Sulochana *et al*., 2016).

Considering that gastric mucosa is continuously exposed to endogenous and exogenous aggressive agents, it is remarkable that the gastric microcirculation has also been intimately implicated in the maintenance of mucosal integrity. In this regard, mucosal blood perfusion is physiologically dependent of NO, an important vasodilator that also mediates the stimulation of gastric mucus secretion, increasing both endogenous protective factors (Kato, Kitamura, Korolkiewicz, & Takeuchi, 1998). In the ethanol-induced ulcer model, the pretreatment of animals with L-NAME, a NO synthase inhibitor, completely abolished the gastroprotective effect of both SDI (191 mg/kg) and L-arginine, a precursor of endogenous NO synthesis (200 mg/kg), including the mucus and GSH depletion (Figure 5). Again, under conditions of oxidative stress observed in ulcerogenic process, dietary flavonoids founded in SDI may increase NO production and protect its

inactivation. These results could explain that endogenous NO is involved in the gastroprotection promoted by SDI.

Epidemiologic studies indicate that patients treated with non-selective nonsteroidal anti-inflammatory drugs (NSAIDs) have a higher risk of develop gastric ulcers (Drini, 2017). Indomethacin is a non-selective NSAID, prescribed for a variety of inflammatory pathologies, including arthritis. Despite its therapeutic effects, NSAIDs can cause bleeding, ulceration and stomach perforation, as consequence of cyclooxygenase isoenzymes COX-1 and COX-2 inhibition that are responsible for the production of gastroprotective prostaglandins (PGE_2 and PGI_2) (Wallace, 2008). The results depicted in Figure 6A reveals that the SDI (191 mg/kg, p.o.) and PGE_2 (20 μg/kg) reduced significantly the indomethacin-induced gastric ulcers in 71.51 and 77.22 %, respectively, when compared with the vehicle group (V: 9.13 ± 2.17 mm^2, SDI: 2.60 ± 0.63 mm^2). Again, the gastroprotective effect of SDI also seems to be related to the maintenance of protective factors, since SDI inhibited the depletion of mucus and glutathione levels in 47.86 and 33.17 % respectively, when compared to the vehicle group (V: Mucus: 2369.41 ± 214.40 μg alcian blue/g of tissue; GSH: 1719.29 ± 109.53 μg GSH/g of tissue) (Figure 5B-C). In sharp contrast, Baracho and coworkers (2014) observed that the aqueous extract of *Sedum dendroideum* leaves worsening the gastric ulcers induced by indomethacin, suggesting that bálsamo *per se* could induces gastritis. However, it is important to mention that the authors were not able to determine the main components neither stablish the doses of aqueous extract employed in this study.

Moreover, it was addressed if SDI could affect the gastric acid secretion. Here, we observed that SDI administration in animals with gastric hypersecretion induced by pylorus ligature did not alter volume, pH or total acidity of gastric secretion when compared with vehicle control group (V: volume: 7.257 ± 0.461 mL; pH: 1.742 ± 0.07;

Total acidity: 0.0612 ± 0.0032 mEq[H^+]/mL) (Figure 7). As expected, the positive control of the test, omeprazole decreased the secreted volume, pH and total acidity, probably due its mechanism of action, represented by the inhibition of proton pumps in the parietal cell ducts (O: Volume: 4.429 ± 0.366 mL; pH: 6.99 ± 0.119; Total acidity: 0.0304 ± 0.0034 mEq[H^+]/mL). Among medicinal plants popularly used as infusion to treat gastric complains, we found that the infusion of *Curatella Americana*, which is rich in flavonol glycosides such as quercetin, does not exert gastroprotection by antisecretory mechanisms (El-Azizi *et al*., 1980; Hiruma-Lima *et al*., 2009). In addition, quercetin *per se* showed antioxidant effects and protected gastric mucosa against indomethacin-induced ulcers (Alkushi & Elsawy, 2017).

Moreover, our results are partially in accordance with previous studies obtained with the hydroethanolic extract of *Sedum dendroideum* (Carrasco *et al.*, 2014). The authors observed no signs of toxicity and a significantly inhibition of gastric ulcers which was accompanied by the prevention of gastric mucus levels in the ethanol- and indomethacin-induced injury, with lower doses of hydroethanolic extract (25, 50 and 100 mg/kg) when comparing to the SDI (ED_{50}: 191 mg/kg). On the other hand, the antiulcer effect of hydroethanolic extract of *Sedum dendroideum* is not dependent of NO and was attributed to the antisecretory activity.

Interestingly, using the hydroethanol as solvent, Carrasco and coworkers (2014) found the presence of flavonoids (quercetin, rutin, and luteolin), phenols, and tannins, whereas SDI prepared in according to the prepare traditional, using water as solvent yield mostly sugars, phenols and flavonol glycosides (myricetin, quercetin and kaempferol), proving that different solvents yield different composition of secondary metabolites. Additionally, the phytochemical constituents of SDI may also play an essential role in the observed results. Interestingly, flavonoids, kaempferol and quercetin, the main

constituents found in SDI infusion, have been previously studied in several ulcer models as possible gastrointestinal protective agents (Li *et al.*, 2018; Kahraman *et al.*, 2003). Moreover, we demonstrated that a polysaccharide fraction, constituted by a homogalacturonan and a homogalacturonan branched by side chains of arabinans and type II arabinogalactans, obtained and isolated from SDI also promotes gastroprotection (de Oliveira et al., 2018). This pectic polysaccharide reduced ethanol-induced gastric ulcers in rats through preservation of mucus barrier and GSH levels in gastric tissue. In this sense, in addition to the secondary metabolites, primary metabolites as polysaccharides are also involved in the gastroprotective effects of SDI.

Thus, it is evident that SDI presents mixtures of active compounds that could act synergistically to exert the antioxidant and antiulcer effects observed in our study. Therefore, it seems unlikely that such a broad spectrum of mucosal protection as that exerted by SDI depends on a unique mechanism of action, but surely is not associated with antisecretory effects, different from the results obtained by Carrasco and coworkers (2014) using hydroethanol extract, where it was demonstrated reduction of the gastric secretion and of the stomach ph. Thus, SDI could offer a safety treatment option when compared with reference drugs, such as omeprazole. Recent observational studies have associated the long-term use of PPIs with some unwanted effects, like nutritional deficiency related to malabsorption of nutrients, risk of bone fracture and risk of *Clostridium difficile* enteric infections, all related to the modification of stomach and intestinal pH (Nehra, Alexander, Loftus, & Nehra, 2018).

The pathogenesis of peptic ulcer disease involves multiple causes, for instance, the exogenous factors which have already been considered, genetic factors and endogenous factors, such as pathophysiological disorders, including abnormal motility and gastric empty (Quigley, 2017). SDI did not display gastric emptying effects in mice

when compared to the vehicle (V: 11.13 ± 1.82%) (Figure 8A). However, it is well known that prokinetics drugs are used to relief the gastric symptoms. Therefore, natural products, like SDI, may be an interesting alternative, once the pretreatment of mice increased the intestinal motility in 56.99 and 66.90% at doses of 150 and 300 mg/kg, respectively, when compared to the vehicle group (V: 40.27 ± 2.37%) (Figure 8B). Regarding the mechanisms underlying the increase of the small intestinal transit promoted by SDI, it was obviously that both atropine, a muscarinic receptor antagonist, and loperamide, a μ-opioid receptor agonist, inhibited intestinal motility. However, the prokinetic effect of SDI 150 mg/kg was blunted in animals treated with atropine and loperamide, decreasing in 30.88 and 60.76% the intestinal transit, respectively, when compared to vehicle group (V: 46.84 ± 3.17%) (Figure 8C). Altogether, our data indicates that SDI could increases peristalsis through cholinergic pathways signaling, once atropine directly blockade the cholinergic transmission and loperamide through the activation of μ-opioid receptors has the same effect, decreasing the peristalsis and consequently the intestinal motility, reversing the prokinetic effect of SDI. Considering the constituents characterized in SDI, is reasonable suggest that both polyphenolic compounds and pectic polysaccharides could improve the digestive health (de Oliveira et al., 2018; Ammar et al, 2018; Wang et al, 2018).

4. Conclusion

Collectively, our results show that *Sedum dendroideum* tea infusion prepared in according to the ethenopharmacological use contains several phenolic components with antioxidant properties, specially flavonols as quercetin, myricetin and kaempferol, and their glycosides due to aqueous extraction (infusion). These natural antioxidant compounds promote beneficial effects on the gastrointestinal tract *in vivo*, preventing the formation of lesions, without altering gastric acid secretion and no apparent signs of

toxicity in colonic cells in vitro. This detailed mechanistic study provided a scientific basis for use of *Sedum dendroideum* in traditional prepare form of the plant, evidencing the unquestionable gastroprotective effect of this plant, confirmed also by others cited works, highlighting as a medicinal plant with promising bioactivity to prevent gastric complaints.

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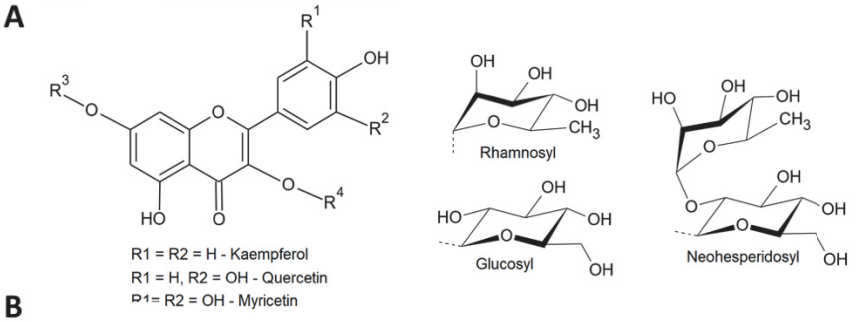
7. Tables and figures

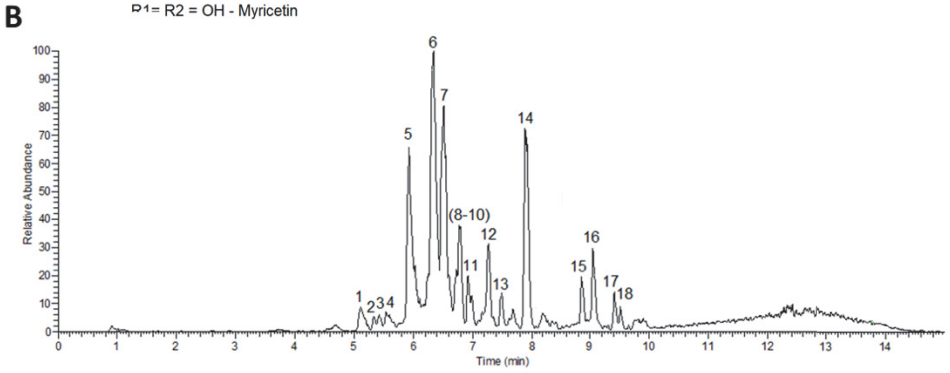
Table 1 – Phytochemical composition of SDI obtained by LC-MS/MS analysis

PEAK	RT	MS^1(-)	MS^2(-)	TENTATIVE IDENTIFICATION
1	5.11	479.1	317.0, 316.0	Myricetin-hexoside
2	5.34	739.4	593.2, 430.1, 429.1, 285.0, 284.0	kaempferol 3-*O*-neohesperidoside 7-*O*-rhamnoside
3	5.43	609.3	463.1, 462.1, 317.0, 315.0	myricetin 3-*O*-rhamnoside 7-*O*-rhamnoside
4	5.53	609.3	463.1, 447.1, 446.1, 301.0, 300.0, 299.0	quercetin 3-*O*-glucoside 7-*O*-rhamnoside
5	5.93	593.2	447.1, 431.2, 430.1, 285.1, 284.1	kaempferol 3-*O*-glucoside 7-*O*-rhamnoside
6	6.34	577.3	431.1, 430.1, 285.0	kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside
7	6.51	737.3	675.3, 635.3, 593.3, 471.3, 429.1, 284.1	* kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside
8	6.69	577.3	431.1, 285.0	Kaempferitrin (isomer)
9	6.72	693.3	577.2, 431.1, 285.0	* kaempferitrin
10	6.77	591.3	529.1, 489.1, 447.2, 285.1, 284.0	* kaempferol glucoside
11	6.92	431.2	285.0, 284.0	kaempferol 3-*O*-rhamnoside
12	7.26	539.2	477.2, 437.2, 395.3, 377.1, 305.2, 275.0	n.i.
13	7.50	395.2	305.2, 275.1	n.i.
14	7.90	431.2	285.0, 284.0	kaempferol 7-*O*-rhamnoside
15	8.85	793.3	635.3, 593.2, 575.3, 471.3, 284.0	* kaempferol diglycoside
16	9.04	327.3	291.2, 229.2, 211.2	n.i.
17	9.40	329.3	293.2, 229.1, 211.1	n.i.
18	9.52	327.2	309.2, 291.2, 273.2, 201.2, 171.0	n.i.

n.i. = not identified

* = structures containing a not identified group linked to flavonol-glycoside





C

	Phenolic content	Flavonoid content
Sedum dendroideum Infusion (SDI)	2.30 ± 0.21 g/100 g	1.12 ± 0.06 g/100 g

Fig. 1. Phytochemical analysis of SDI. Major flavonoids constituents in SDI (Panel A). HPLC–MS (high performance liquid chromatography coupled with mass spectrometer) profile of the SDI (Panel B). Quantification of total phenolic and flavonoid contents in SDI (Panel C).

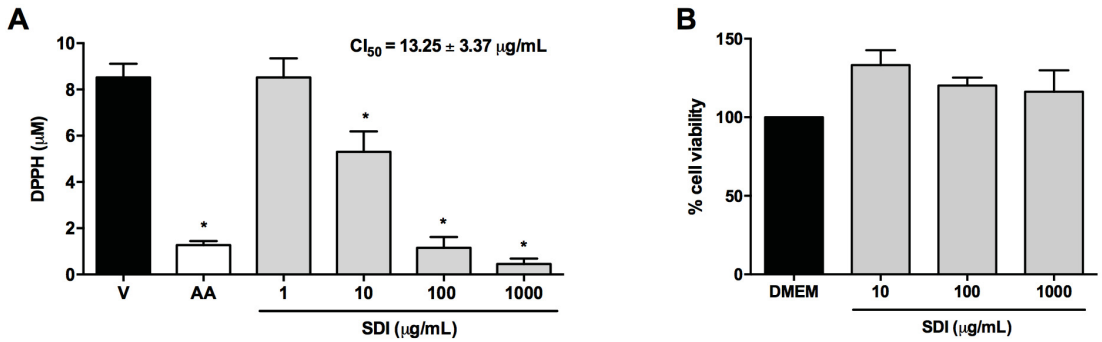


Fig. 2. Effects of SDI on DPPH radical scavenging activity (Panel A) and on Caco-2 cell viability (Panel B). Data on graph are representative of experiments performed at least three times in triplicate. *P< 0.05, One way ANOVA followed by Bonferroni post hoc test.

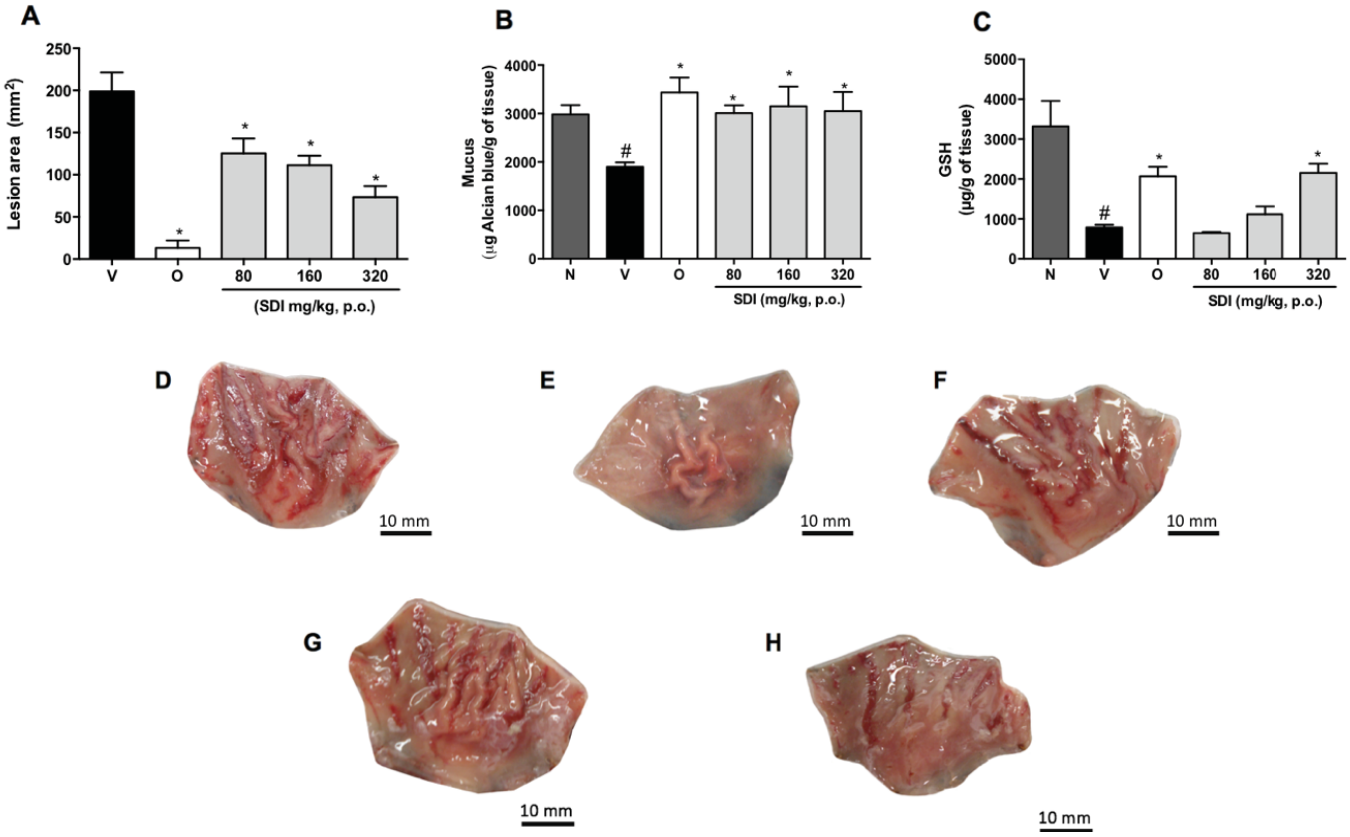


Fig. 3. Effect of oral pretreatment with SDI on gastric lesions induced by ethanol in rats.The panel A shows the gastric ulcer area (mm2); B mucus content (µg Alcian Blue/g of tissue) and C gluthatione levels (µg GSH/g of tissue). Panels D (Vehicle), E (Omeprazole), F (SDI: 80 mg/kg), G (SDI: 160 mg/kg) and H (SDI: 320 mg/kg) are representative of macroscopic photograph of stomachs. Results are expressed as mean ± S.E.M. *P <0.05 when compared to vehicle group (V), #P<0.05 when compared to naïve group (N).

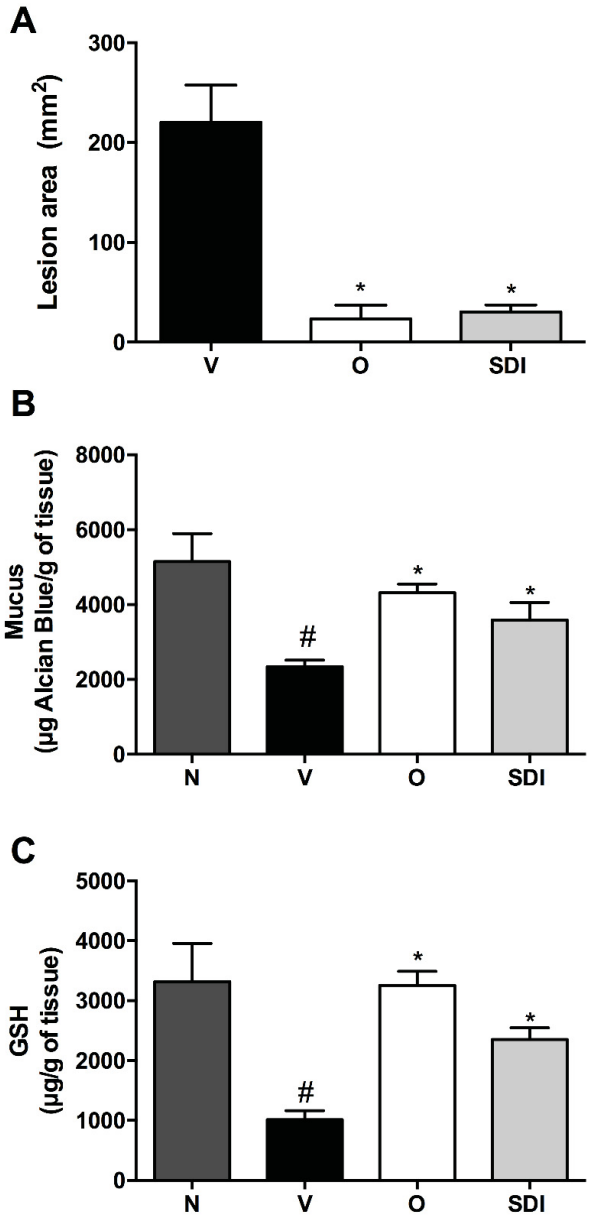


Figure 4. Effect of intraperitoneal pretreatment with SDI on gastric lesions induced by ethanol in rats. The panel A shows the gastric ulcer area (mm2); B mucus content (µg Alcian Blue/g of tissue) and C gluthatione levels (µg GSH/g of tissue). The results are expressed as mean ± S.E.M. $^{*}P < 0.05$ when compared to vehicle group (V), $^{\#}P < 0.05$ when compared to naive group (N

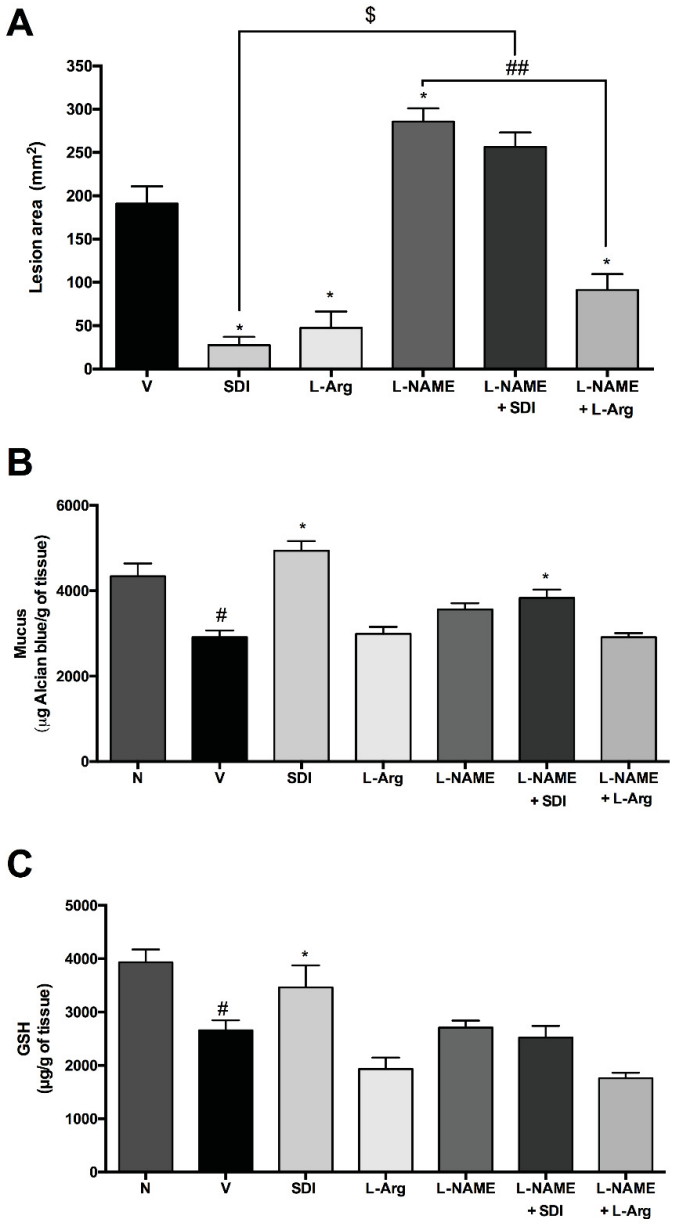


Figure 5. Influence of L-NAME pretreatment on gastroprotection promoted by SDI on gastric lesions induced by ethanol in rats. The panel A shows the gastric ulcer area (mm2); B mucus content (µg Alcian Blue/g of tissue) and C gluthatione levels (µg GSH/g of tissue). The results are expressed as mean ± S.E.M. $*P < 0.05$ when compared to vehicle group (V). $^{\$}P<0.05$ when compared to Sedum dendroideum infusion group (SDI). $^{\#\#}P<0.05$ when compared to L-NAME group (L-NAME). $^{\#}P<0.05$ when compared to naive group (N).

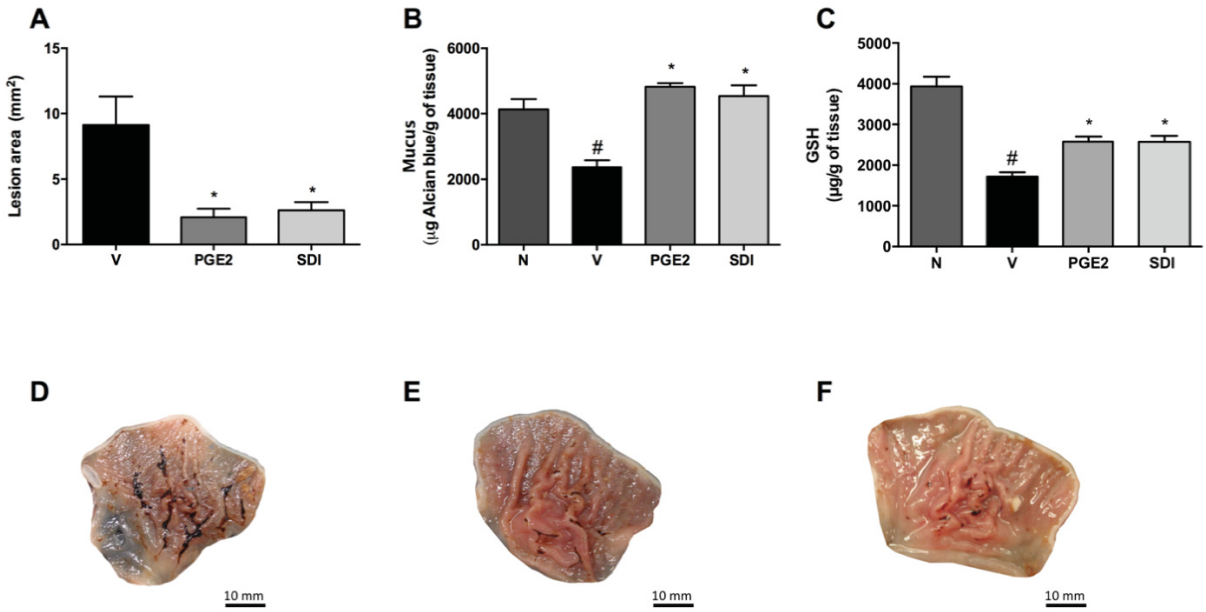


Figure 6. Effect of oral treatment with SDI on gastric lesions induced by indomethacin in rats. The panel A shows the gastric ulcer area (mm^2); B mucus content (µg Alcian Blue/g of tissue) and C gluthatione levels (µg GSH/g of tissue). Panels D (Vehicle), E (Prostaglandin E2) and F (SDI: 191 mg/kg) are representative of macroscopic photograph of stomachs. The results are expressed as mean ± S.E.M. *P <0.05 when compared to vehicle group (V). #P<0.05 when compared to naive group (N).

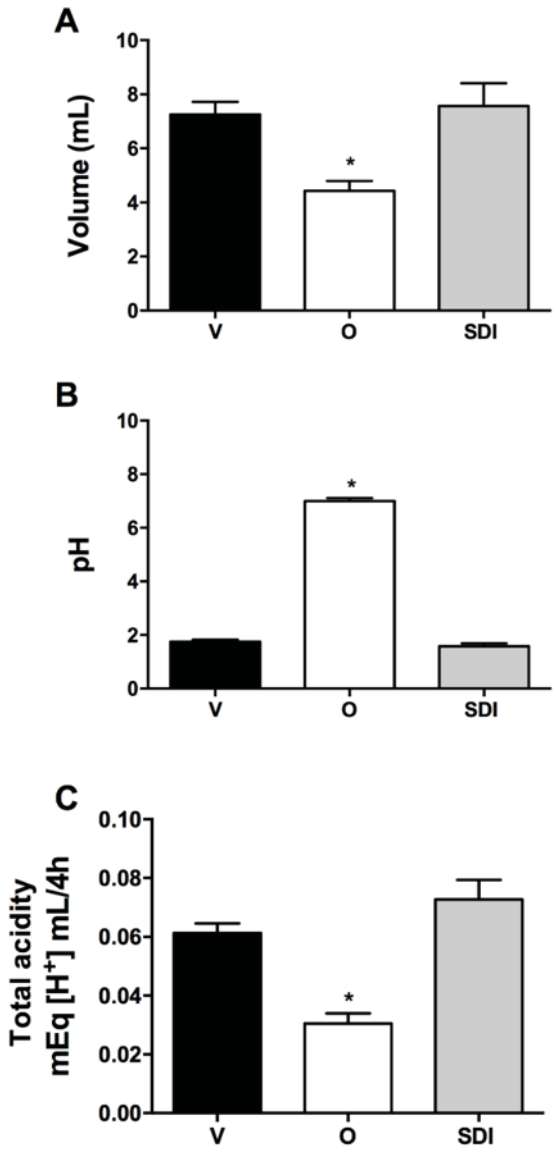


Figure 7. Effect of oral treatment with SDI on the gastric secretion by pylorus ligation in rats. The panel A shows the volume (mL); B pH and C total acidity (mEq [H^+] mL/4h). The results are expressed as mean ± S.E.M. $^*P < 0.05$ when compared to vehicle group (V).

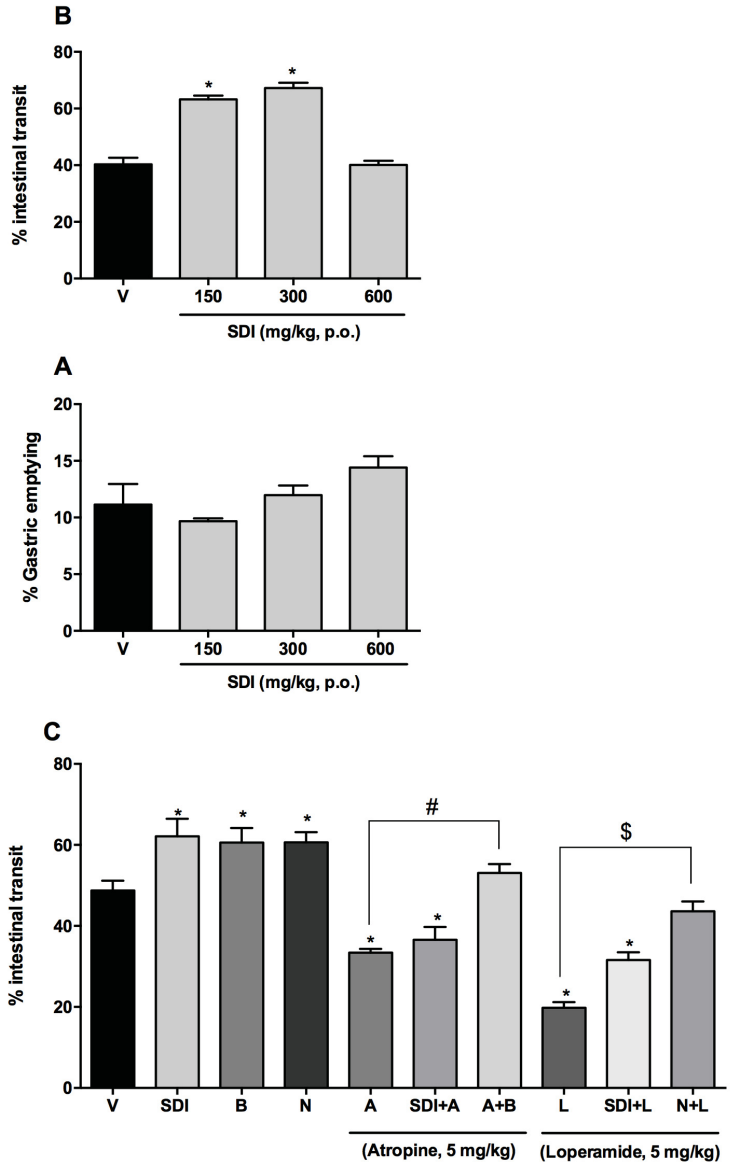


Figure 8. Effect of oral treatment with SDI on the gastric emptying and intestinal motility in mice. The panel A shows the % gastric emptying, B % of intestinal motility and C the mechanism of action underlies SDI effect on % of intestinal motility. Treatments groups are as follows: Water (V), *S. dendroideum* infusion (SDI), Betanecol (B), Naloxone (N), Atropine (A), Loperamide (L). The results are expressed as mean ± S.E.M. *P <0.05 when compared to vehicle group (V). #P<0.05 when compared to SDI group (SDI). $P <0.05 when compared to Loperamide group (L).

4. ARTIGO CIENTÍFICO 2

RESEARCH ARTICLE: Journal of Ethnopharmacology

4.1 *Sedum dendroideum* infusion accelerates the healing of pre-existing gastric ulcers in rats: analysis of underlying mechanisms

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Abstract

Ethnopharmacological relevance

Sedum dendroideum Moc. & Sessé ex DC. (Crassulaceae), popularly known as "bálsamo", is an edible plant employed in Brazilian folk medicine as salad, juice or infusion for the treatment of gastric ulcers and inflammatory diseases. However, the mechanisms of action underlying the gastric healing effects are not yet investigated.

Aim of the study

Our group has already demonstrated that *Sedum dendroideum* infusion (SDI) leaves, rich in phenolic compounds and flavonoids shows gastroprotective effects against acute ulcer models. The present study investigates the healing effects of SDI in chronic gastric ulcer model, and further mechanisms of action underlying this effect.

Materials and methods

The healing property was analyzed in the 80% acetic acid-induced chronic gastric ulcers. Rats were orally treated with vehicle (water, 1 mL/kg), SDI (191 mg/kg), omeprazole (40 mg/kg) or sucralfate (100 mg/kg) twice daily for 5 days after ulcer induction. Following treatments, toxicological effects, macroscopic ulcer appearance, microscopic histological (HE, mucin PAS-staining) and immunohistochemical (PCNA) analysis, inflammatory (MPO and NAG activity, cytokine levels measurements) and antioxidant (SOD and CAT) parameters were investigated in gastric ulcer tissues.

Results

The animals did not present any signs of toxicity, evaluated through body weight, ratio organ/body weight and ALT, AST, creatinine and urea measurements. SDI accelerated

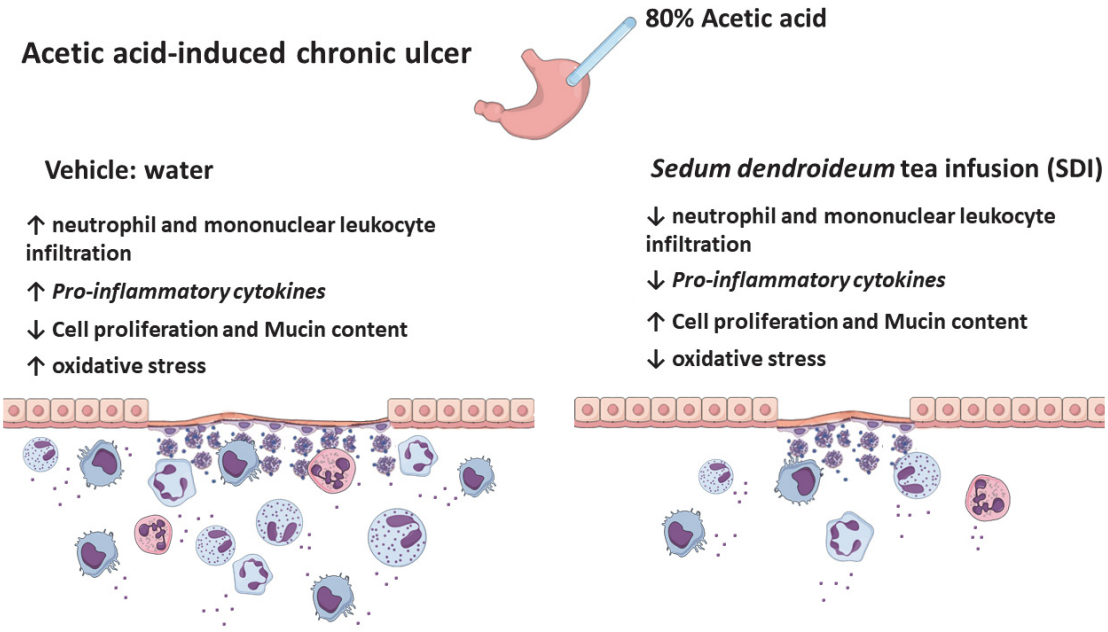
gastric ulcer healing, through maintenance of mucin contents and gastric epithelial cell proliferation. Moreover, SDI reduced neutrophil and mononuclear leukocyte infiltration, TNF-α and IL-1β levels in inflammatory site, as well as the oxidative stress, restoring SOD and CAT activities. All these data were found to be comparable with omeprazole and sucralfate treatments.

Conclusions

The gastric healing effect of *Sedum dendroideum* against pre-formed chronic acetic acid induced gastric ulcers is attributed to the reinforcement of mucus protective barrier, increasing of gastric cell proliferation and improvement of the inflammatory and oxidative stress response. Interestingly, we previously demonstrated that SDI effects are different of omeprazole, since it is not related to antisecretory mechanisms. Collectively, our findings demonstrated the effectiveness of SDI used in ethnomedicine, offering therapeutic alternatives for the treatment of gastric ulcer.

Keywords: chronic ulcer; gastroprotection; ethnopharmacological use; antioxidant; anti-inflammatory

Graphical abstract



1. Introduction

Peptic ulcer disease affects 10% of the world population (Zapata-Colindres *et al.*, 2006), and is characterized by a distinct deep necrotic injury resulting in the destruction of stomach mucosal and submucosa layers (Lanas & Chan, 2017). Traditionally, gastric ulcers develop when occurs an imbalance in the equilibrium between protective and aggressive factors of the gastric mucosa. Often is considered as a chronic disease, due to the ulcer recurrence even after total mucosal healing (Yandrapu & Sarosiek, 2015; Kangwan, *et al*., 2014).

The healing of gastric mucosa is a complex process and involves numerous components like growth factors, hormones, and inflammatory cytokines that stimulate cell proliferation and migrating, reepithelization the ulcerated area and finally tissue regeneration (Tarnawski; Ahluwalia, 2012).

Sedum dendroideum is a succulent plant widely employed in Brazilian folk medicine for the treatment of gastric ulcers and inflammatory diseases (De Melo *et al*., 2005, Carrasco et al., 2014). In a previous study, our group demonstrated that an infusion prepared by soaking the leaves in hot water has prokinetic and gastroprotective effects against acute gastric lesions induced by ethanol and indomethacin, without changes in gastric acid secretion (Da Luz et al., 2018, submitted). Recently, several studies have suggested that the use of gastric acid suppressive medications for long time features side effects like nutritional deficiency related to malabsorption of nutrients, risk of bone fracture and risk of *Clostridium difficile* enteric infections, all related to the modification of stomach and intestinal pH (Vaezi *et al*., 2017; Naito *et al*., 2018).

Therefore, new therapeutic alternatives using medicinal plants have been received great attention, since it could decreased these undesirable effects. Among the plethora of brazilian traditional plants used for the treatment of peptic ulcer, we highlight *Schinus*

terebinthifolius, *Maytenus ilicifolia* and *Citrus aurantium*, which were successfully studied and that had their potential of medicinal market plants explored (Baggio et al., 2007; Carlini et al., 2010, Polo et al., 2012)

Considering the ethnopharmacological profile, *Sedum dendroideum* emerge as an important plant that deserves further investigation. Nevertheless, despite the healing ulcer property of *Sedum dendroideum* have been previously demonstrated (Carrasco et al, 2014), the mechanisms responsible for its ulcer cicatrization have not yet been adequately elucidated. In this context, we have investigated the mechanisms involved in the ulcer healing activity of *Sedum dendroideum* leaves infusion (SDI), rich in phenolic and flavonoids constituents, in a chronic gastric injury model.

2. Materials and methods

2.1. Botanical material and infusion preparation

The infusion of *Sedum dendroideum* leaves (SDI) were obtained as previously described (De Oliveira et al., 2018; Da Luz et al., 2018, submitted). In brief, the plants was harvested in Campina Grande do Sul (25°19'05.3" S; 49°02'32.3" W, at 921m above mean sea level), State of Parana (PR), South of Brazil and identified by Dr. José Tadeu Weidlich Motta at Municipal Botanical Museum (MBM) of Curitiba, PR, Brazil (MBM-272917). The SDI was prepared by extraction with boiling water (100 g/L) by infusion during 1 h, followed by lyophilization process in order to obtain a dry extract. The SDI dose employed in this study (191 mg/kg, p.o.) was obtained as the median effective dose (ED50) based on inhibition of gastric lesion induced by ethanol (Da Luz et al., 2018, submitted). It is important to highlight that the choice of study the gastroprotective effects only of the ED50 dose was taken according to the ethnopharmacological use of *Sedum dendroideum* infusion, following an allometric scaling approach, normalizing the human

dose for rats (Nair and Jacob, 2016). Furthermore, this approach was undertaken aiming to reduce the number of experimental animals, in agreement to the 3Rs principles.

2.2. Animals

Female Wistar rats, obtained from the Biotery of Federal University of Parana weight 180 to 200 g, were kept in plastic cages containing pine bedding (maximum of 5 rats per cage) and maintained at 22 ± 2 ºC and 12 hours - light/dark cycle, with food (Nuvi-Lab CR-1, Quimtia S/A, Colombo, PR, Brazil) and water *ad libitum*. All animal protocols were approved by the Committee of Animal Experimentation of Federal University of Paraná (CEUA/BIO - UFPR: nº 1010) and conducted in agreement with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (2011).

2.3. Chronic gastric ulcer induced by acetic acid

To evaluate the gastric ulcer healing, the chronic gastric ulcers were induced with acetic acid as described previously, with modifications (Okabe et al., 1971). Rats were anesthetized with a combination of xylazine and ketamine (10 and 5 mg/kg, *i.p.* respectively) and after a laparotomy, the stomach was exposed and a cylinder (6 mm of diameter) containing 500 µL of a solution of 80% glacial acetic acid was applied to the serosal surface of the stomach. After 1 min, acetic acid was aspirated, the stomach washed with sterile saline and replaced, abdomen was sutured and then rats returned to their cages. Following the surgery, animals were fasted for 24 h and on the second day after ulcer induction, they received restricted feed twice a day for 1 h until the end of experiment. Animals were orally treated twice daily 1 h after feeding, with water (Vehicle, V: 1 ml/kg), omeprazole (O: 40 mg/kg), sucralfate (100 mg/kg) or with SDI

(191 mg/kg) for 5 days. On the day following the last treatment, rats were euthanized by thiopental overdose (100 mg/kg, i.p.) and the stomach was removed and opened for calculate the ulcer area (mm^2), measured as length (mm) × width (mm).

2.4. Histology evaluation

Stomach histology was performed to evaluate microscopic damage induced by acetic acid. The ulcerated gastric tissue was fixed in 10% formaldehyde, dehydrated with alcohol and xylene and embedded in paraffin wax. After that, ulcer tissue was cut into 5-μm sections on a microtome, placed on gelatin-coated microscope slides and stained with hematoxylin/eosin (HE) for histological evaluation. The ulcers sections were observed and photographed using Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (MetaSystems, Altlussheim, DE) at at ×20 and ×400 magnification.

2.5. Determination of mucin content

Mucin histochemistry was performed to evaluate the alterations on mucus content after acetic acid-induced gastric ulcer, as previously described by Pereira et al., (2013). Paraffin-embedded sections were deparaffinized, rehydrated, oxidized in 0.5% periodic acid for 5 min and washed in distilled water. After, the sections were stained with Schiff's reagent for 20 min and subsequently washed with sulphurous water (three times of 2 min) and with tap water for 10 min. Lastly, ulcer sections were counterstained with hematoxylin for 20 s and dehydrated. Periodic acid-Schiff (PAS) mucin staining positive pixels patterns were quantified with ImageJ® software.

2.6. Determination of gastric epithelial cell proliferation

Proliferating cell nuclear antigen (PCNA) was used to determine by immunohistochemical staining cell proliferation in gastric mucosa after acetic acid-induced gastric ulcer. Following deparaffinization/rehydration protocols, sections were processed for antigen unmasking (10 mM sodium citrate buffer, pH 6.0 at 95 °C), endogenous peroxidase activity blocked (3% H_2O_2/methanol for 15 min) and to prevent non-specific binding of the antibody to the tissues (1% BSA, 0.3% Triton X-100, 1X PBS for 45 min). Then, sections incubated overnight at 4 ºC in a humidified chamber with goat anti-PCNA (at 1:100; Santa Cruz Biotechnology Inc., CA, USA), rinsed in PBS and finally incubated with a HRP secondary antibody (1:100, Santa Cruz Biotechnology Inc., CA, USA) at room temperature for 1 h. After washing, the peroxidase-binding sites were detected by staining with DAB substrate Kit (BD Biosciences, California, USA), counterstained with hematoxylin, dehydrated and mounted with coverslip with permanent mounting medium (Entellan® Merck, Darmstadt. Germany). PCNA-positive cells were quantified in representative areas with 400x magnification in an optical microscope, performed by counting the number of positive cells per field of three fields from three different histological sections of three animals per group.

2.7. Preparation of subcellular fractions of stomachs

The samples of stomach were homogenized with cold 200 mM potassium phosphate buffer (pH 6.5) and centrifuged at 9000 × *g* for 20 min at 4 ºC, the supernatant was used for the determination of superoxide dismutase (SOD), catalase (CAT) and the pellet was used for evaluated the myeloperoxidase (MPO) levels and -acetil-β-D-glicosaminidase (NAG) activity. The protein concentrations of the samples were

determined by the Bradford method (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as standard (0.062 - 1 mg/mL).

2.8. Measurement of myeloperoxidase (MPO) activity

Infiltration of neutrophils in the gastric ulcers was measured through of MPO activity according to the method described by Bradley et al. (1982). The pellet was resuspended in 80 mM potassium phosphate buffer (0.5% hexadecyltrimethylammonium bromide (HTAB), pH 5.4) and centrifuged at 11.000 × *g* for 20 min at 4 °C. The supernatant was mixed with buffer (0.08 M and 0.22 M phosphate buffer, 0.017% H_2O_2) and the colorimetric reaction was obtained with 18.4 mM 3,3′, 5,5′-tetramethylbenzidine (TMB) on a 96-well plate. Absorbance of the samples was determined at 620 nm, and the results expressed as units of optic density (O.D.)/mg of protein.

2.9. Measurement of N-acetyl-β-D-glucosaminidase (NAG) activity

NAG enzymatic activity was used to correlate the presence of mononuclear leukocyte in the gastric ulcers. Samples of the supernatant were incubated with citrate buffer (50 mM, pH 4.5) in the presence of 2.24 mM 4-Nitrophenyl N-acetyl-β-D-glucosaminide. The 96-well plate was incubated at 37 °C for 60 min and then 200 mM glycine buffer, pH 10.4, interrupted the reaction. Absorbance of the samples was determined at 405 nm, and the results expressed as units of optic density (O.D.)/mg of protein.

2.10. Pro-inflammatory cytokine measurements

Gastric ulcer samples were homogenized with ice-cold RIPA-buffer (1 mM Tris-HCl pH 7.5, 5 M NaCl, 0.5 M EDTA) with protease and phosphatase inhibitors (10 μL/mL), centrifuged at 9.000 × *g* for 20 min. Supernatants were used to estimate the tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β levels, using commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (Peprotech EC Ltd, London UK). The absorbance for TNF-α and IL-1 β was measured at 405 nm with wavelength correction set at 650 nm and at 450 nm with wavelength correction set at 620 nm, respectively. Recombinant rat TNF-α standard curve (31.2–3000 pg/mL) and recombinant rat IL-1β standard curve (7.8 – 4000 pg/mL) were used to interpolate concentrations of all the samples. All results were expressed in pg/mg of protein.

2.11. Determination of superoxide dismutase (SOD) activity

The method used to determine SOD activity is based in the capacity of SOD to inhibit pyrogallol autoxidation (Halliwell; Gutteridge; Grootveld, 1985), Pyrogallol (1 mM) was mixed with buffer solution (200 mM Tris HCl–EDTA, pH 8.5) and supernatant aliquots, and then vortexed for 1 min. The reaction was incubated for 20 min at room temperature and stopped with 1 N HCl. The absorbance was measured at 405 nm using a microplate reader. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U/mg of protein.

2.12. Determination of catalase (CAT) activity

Catalase activity was determined based in the decomposition of H_2O_2, according Aebi (1984). In plate of 96 wells, supernatant aliquots were mixed with reactive solution (1 mM Tris EDTA; 5 mM EDTA and 30% of H_2O_2). The absorbance was measured at 240 nm for 1 min using a microplate reader. CAT activity was expressed in $mmol/mL/min^{-1}$.

2.13. Evaluation of toxicity

During the treatment period, the body weight of rats were recorded daily and the animals were observed for detection of undesirable clinical and behavioral signs. At the end of the treatment, the animals were sacrificed as previously described, and the selected organs were removed and weighted (adrenal, heart, kidney, liver, lung, spleen, ovaries and uterus). Organ weights were measured and are reported relative to the body weight. Following cardiac puncture, plasma was obtained after centrifugation of blood at 4000 X g for 5 min and was assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (marker of liver damage), and creatinine and urea (marker of kidney injury). The parameters were analyzed by an automated system (Mindray BS-200) according to the kit manufacturer's instructions (Labtest Diagnostica, Brazil).

2.14. Statistical analysis

Results were expressed as mean ± standard error of mean (SEM) and statistical differences between experimental groups was determined using one-way analysis of variance one-way ANOVA followed by Bonferroni's multi-comparison post-hoc test, all analyzes were performed with GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA). Differences were significant when $P \leq 0.05$.

3. Results and discussions

Our results indicate that the oral treatment twice daily with infusion of *Sedum dendroideum* leaves promotes the ulcer healing, decreasing several parameters related to the inflammatory response and oxidative stress.

In rats, gastric ulcer model induced by acetic acid are very similar to those observed in humans, resembling characteristics of healing and recurrence. Evaluation of histological section demonstrated a penetrating ulcer associated with destruction of gastric mucosa and muscle tissue, together with the formation of leukocyte exudate, edema and cellular infiltration (Okabe & Amagase, 2005). After five days of treatment, SDI was able of accelerated the healing of chronic gastric ulcer in 36.64% when compared to vehicle group (194 ± 12.50 mm^2) (Fig. 1). The pharmacological therapy for chronic treatment of gastric ulcers involve drugs that enhance mucosal protection or that decreased aggressive factors (Tang & Chan, 2012). Consequently, we select as positive controls drugs with different mechanisms of action, such as omeprazole, which inhibits the gastric acid secretion and as sucralfate, which display gastroprotective effects by creating a mechanical barrier. In relation to the positives controls, omeprazole and sucralfate and reduced significantly the ulcer area in 42.74 and 42.57% (S: 111.41 ± 8.97 mm^2; O: 111.08 ± 3.24 mm^2) (Fig. 1).

The evaluation macroscopic and histological slices of gastric ulcers confirm the gastric mucosal injury caused by acetic acid (Fig 2, Panels A and E) and the healing of the ulcer area when treated with omeprazole (40 mg/ kg), sucralfate (100 mg/kg) and SDI (191 mg/kg) (Fig. 2, Panels B-H). Histological evaluation also showed that the instillation of acetic acid in the serosa of the stomach promotes ulcer development, decreasing the amount of mucin-like glycoproteins in the vehicle group (Fig 3, Panels A and E).

However, the chronic treatment with omeprazole, sucralfate and SDI were able to increase the mucin staining when comparing to the vehicle group in 44.02, 53.74 and 52.96% respectively (Fig. 5, Panel A) (V: 6.35 ± 5.40 pixels/field × 10^4). The first line of defense against acid is the mucus layer, and the presence of mucin-like glycoproteins support the tissue regeneration and prevents further injury during the healing process (Laine; Takeuchi; Tarnawski, 2008).

Complementarily, the immunohistochemical PCNA evaluation showed that chronic treatment with SDI increased relevantly the number of epithelail proliferating cells, as observed in the controls treated groups (Fig. 4, Panel A). Interestingly, it is well known that the ulcer healing process depends of the restoration of mucosal tissue integrity, a fundamental process that implicate in reepithelialization, cell differentiation, migration and proliferation (Tarnawski; Ahluwalia & Jones, 2014). Thus, the treatment with omeprazole, sucralfate and SDI showed an expressive increase of 57.89, 61.15 and 52.95%, respectively, in the number of PCNA-positive cells when compared to vehicle (V: 109.66 ± 3.44) (Fig. 4, Fig. 5 Panel B). These data confirm and reinforce that SDI is able to accelerate the healing of gastric mucosal in the acetic acid-induced chronic injury, a well stablished model of intractable ulcer, which is observed clinically (Kitajima et al., 1993).

Furthermore, the inflammatory response at the ulcer scar site is also deeply related to the quality of ulcer healing. Inflammation involves the recruitment of neutrophils and mononuclear cells, that in turn leads to the increase of intracellular reactive oxygen species as well as triggers pro-inflammatory cytokine production, such as TNF-α and IL-1β (Osawa, 2018). As expected, in the present study, the acetic acid induced-gastric ulcer injury promotes an increase in MPO and NAG enzyme activities in the vehicle treated group, due to the exacerbated inflammatory response in the stomach (Contreras-Zentella

et al., 2017). When compared to the naïve group, MPO and NAG activity increased in 48.81 and 68.30% in vehicle ulcerated group (N: MPO: 1.18 ± 0.02 O.D./mg of protein; NAG: 36.29 ± 1.23 O.D./mg of protein) (Fig. 6, Panel A and B, respectively). The data presented in the Fig. 6, Panel A shows that animals treated with omeprazole, sucralfate and SDI significantly decreased MPO activity in 58.75, 74.22, and 93.02 %, respectively, when compared to vehicle group (V: 3.79 ± 0.69 optic density O.D./mg of protein). Likewise, omeprazole, sucralfate and SDI reduced NAG activity in 32.84, 35.43 and 29.77% when compared to the vehicle group (V: 61.09 ± 5.80 O.D./mg of protein) (Fig. 6, Panel B).

In line with these observations, omeprazole, sucralfate and SDI treatments significantly decrease the gastric TNF-α levels in 52.43, 45.66 and 62.43%, when compared to vehicle group (V: 397.69 ± 70.93 pg/mg of protein) (Fig. 6, Panel C). In addition, gastric IL-1β levels were also reduced by the treatment with omeprazole sucralfate and SDI in 56.87, 64.95 and 57.75% when compared to vehicle group (V: 472.39 ± 50.83 pg/mg of tissue) (Fig. 6, Panel D). Notably, De Melo et al. (2005) found several kaempferol glycosides, being kaempferitrin the most abundant in the fresh juice of *Sedum dendroideum*, and suggest that this chemical profile may explain the its popular use against pain and inflammatory diseases. These results are consistent with our research group's previous findings, where we demonstrate different flavonol glycosides, containing myricetin and quercetin, along with the kaempferol as aglycones in SDI (Da Luz et al., 2018, submitted). Given the excellent anti-inflammatory effect of SDI, the control of the inflammatory cells infiltration and consequent impairment of the cytokines overexpression may contributes significantly to the healing process of gastric ulcers (Furuta et al., 2002, Watanabe et al, 2002).

As mentioned, we had performed the phytochemical investigation of SDI, and found a high content of phenolic compounds and flavonoids (Da Luz et al., 2018, submitted). There is general agreement that flavonoids, a group of polyphenolic compounds, possess both free-radical scavenging and anti-inflammatory properties (De Melo *et al.*, 2009). In this regard, *in vitro* free radical scavenging activity of SDI was previously assessed against DPPH, suggesting that this tea infusion represent a potent body defense against free radicals. In fact, Li and coworkers (2018) also showed that kaempferol, which is present in SDI, has a potent antioxidant effect in animal models.

In physiological conditions, it is known that gastric mucosal is normally a key source of ROS. However, disproportionate oxidative stress is considered a pivotal factor in the pathogenesis and maintenance of gastric ulcers, since the overproduction of ROS and free radicals favors the impairment of stomach antioxidant defense mechanisms (Bhattacharyya et al., 2014). In the stomach, superoxide dismutase, that converts the superoxide radicals into hydrogen peroxide and catalase, that separates hydrogen peroxide into oxygen and water, are considered a major antioxidant defenses against ROS production (Weydert & Cullen, 2010). The gastric ulcer induced by acetic acid increased SOD activity by 55.51% when compared to naïve group (N: 59.86 ± 7.50 U of SOD/mg of protein) (Fig. 7, Panel A). The treatment of animals with omeprazole, sucralfate and SDI were able to significantly reversed, dose-dependent manner, the increase on SOD activity in 19.99, 20.20 and 25.13%, respectively when compared to the vehicle group (V: 135.55 ± 6.85 U of SOD/mg of protein) (Fig. 7, Panel A). The catalase activity was decreased in ulcerated stomach in 39.28%, when compared to naïve group (N: 0.42 ± 0.03 $mmol/ml/min^{-1}$). This data could be explained due to the excessive enzymatic activity to remove free radicals, leading to the catalase depletion during the inflammatory process observed in the chronic ulcer model (Murugan & Pari, 2007). Again, animals treatment

with omeprazole, sucralfate and SDI were able to replace in 61.35, 56.78 and 59.96% the catalase activity, when compared to vehicle group (V: 0.25 ± 0.02 mmol/ml/min^{-1}) (Fig. 7, Panel B). It is worth noting that our results indicates a positive correlation between the reduction of oxidative stress and the healing ulcer promoted by SDI. In addition, in agreement with our data, Adzu *et al.* (2015) shown that leaves infusion of *Copaifera malmei* exhibit similar antioxidant and gastroprotective effects, contributing to reinforce the antioxidant role of SDI to support gastric ulcer healing.

Sedum dendroideum is drunk in the form of infusion as a popular medicine to treat gastric disorders. Interestingly, no toxic effects have ever been reported in the literature in *in vivo* or *in vitro* assays (Carrasco et al., 2014; Da Luz et al., 2018, submitted). In line with this view, following the subchronic treatment with the therapeutic dose of SDI (191 mg/kg), no adverse reactions or toxicity were observed, as well as no differences in body weight or the ratio organ weight/body weight (Fig. 8, Panels A and B). Moreover, at this point, no changes in the serum toxicological parameters were observed. The levels of ALT, AST urea and creatinine were within the reference range (Fig. 8, Panel C), suggesting the safety of *Sedum dendroideum* infusion intake.

Thus, the data presented herein, corroborate and extend the findings of Carrasco et al. (2014), revealing underlying gastroprotective mechanisms displayed by *Sedum dendroideum* in accelerate ulcer healing. Moreover, different of omeprazole, SDI unchanged the gastric acid secretion (Da Luz et al., 2018, submitted). These observations reinforce the notion that the mechanisms whereby *Sedum dendroideum* promotes gastroprotection are distinct from those promoted by antisecretory drugs.

4. Conclusion

In conclusion, the results of the present study confirm and reinforce the popular use of *Sedum dendroideum* as gastroprotective agent. This view is strengthened by gastric ulcer healing promoted by orally administered leaves infusion, which ameliorates the inflammatory process and oxidative stress, maintain gastric mucus and promotes increasing of cell proliferation, without signs of toxicity. Our findings pointed out *Sedum dendroideum* as a useful source of bioactive compounds for the treatment of gastric ulcer disease.

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6. Tables and figures

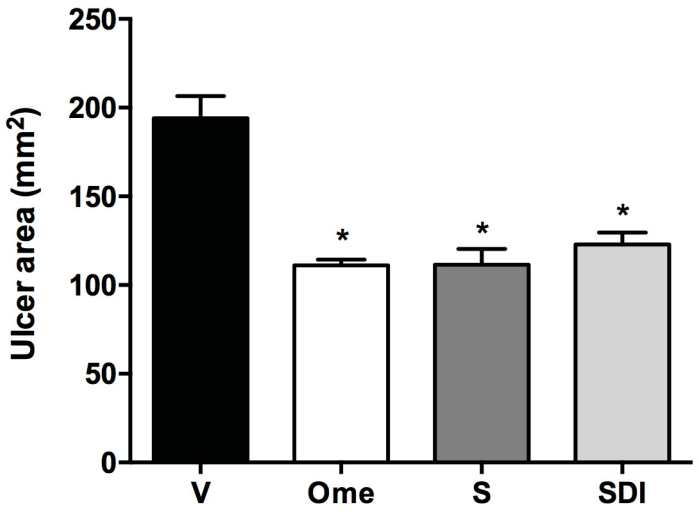


Fig. 1. Effect of *Sedum dendroideum* leaves infusion (SDI) on chronic gastric ulcer induced by 80% acetic acid in rats. The animals were orally treated with vehicle (V: water, 1 mL/kg), omeprazole (Ome: 40 mg/kg), sucralfate (S: 100 mg/kg) or SDI (191 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n = 12). ANOVA followed by Bonferroni's test. *P<0.05 when compared to the ulcerated vehicle group (V).

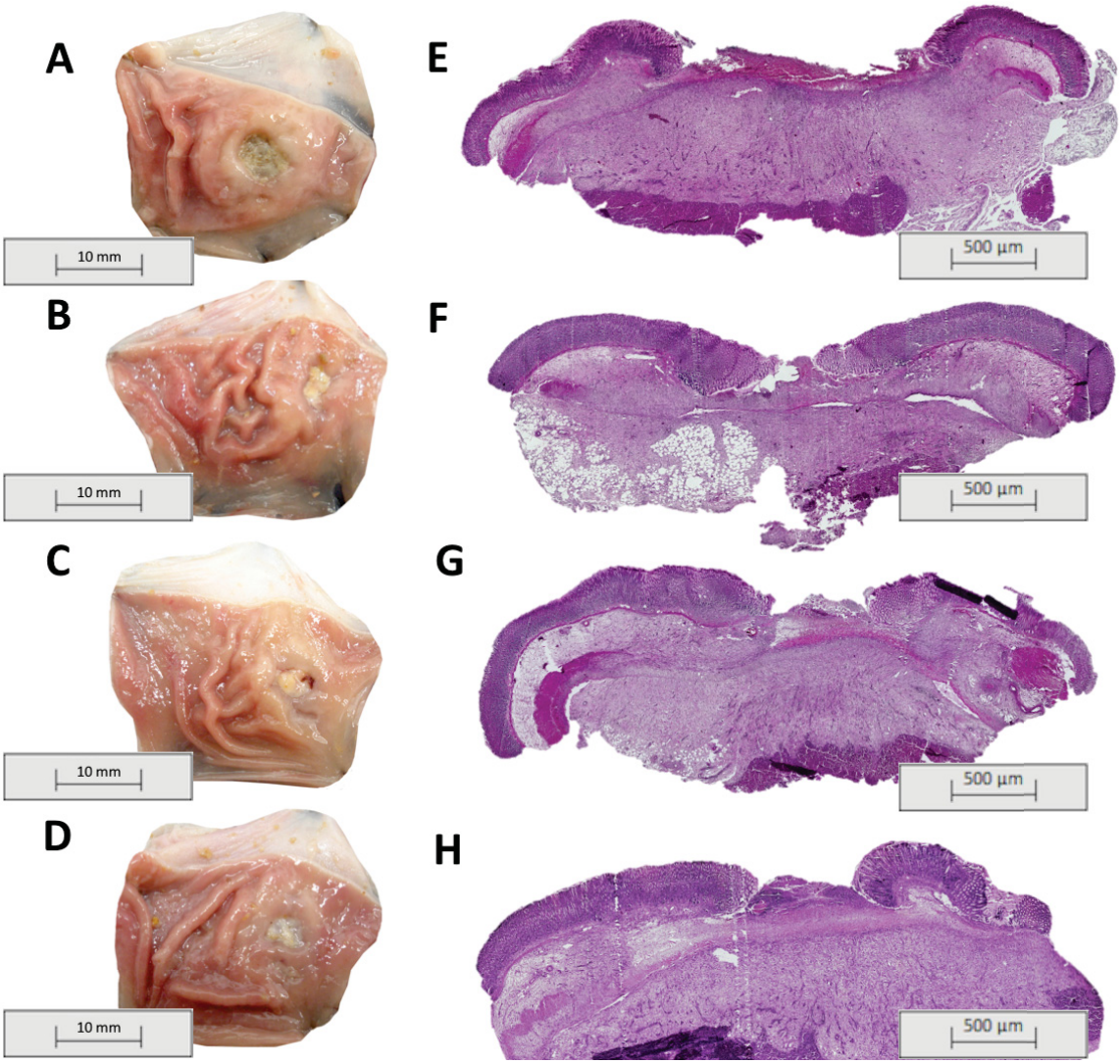


Fig. 2. Effect of *Sedum dendroideum* leaves infusion (SDI) on the regeneration of gastric chronic ulcer induced by 80% acetic acid in rats. The images representing macroscopic photograph (Panels A-D, Bars = 10 mm) and histological hematoxylin/eosin (HE) sections (Panels E – H, Magnification, 20×, Bars = 500 µm) of ulcerated stomachs. The animals were orally treated with vehicle (water, 1 mL/kg, Panels A and E), omeprazole (40 mg/kg, Panels B and F), sucralfate (100 mg/kg, Panels C and G) or SDI (191 mg/kg, Panels D and H) twice daily for five days after the gastric ulcer induction.

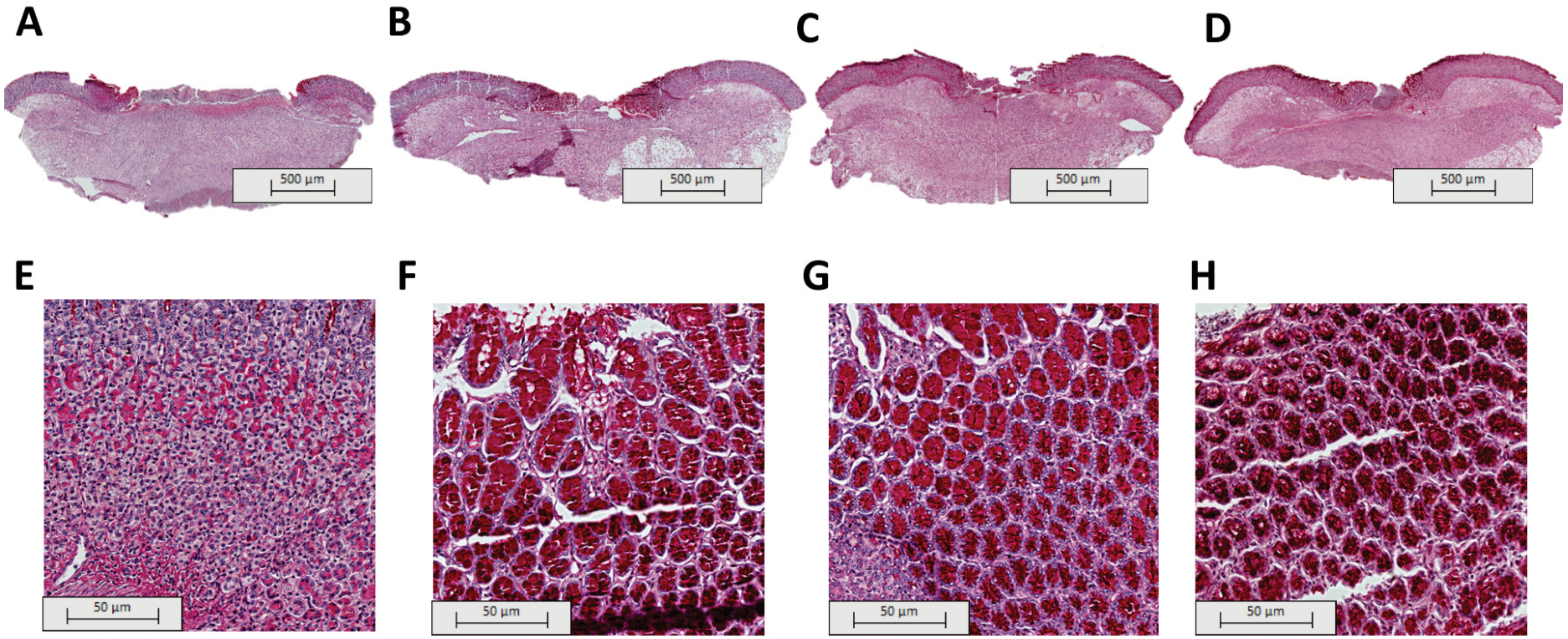


Fig. 3. Effect of *Sedum dendroideum* leaves infusion (SDI) on the histochemical staining for mucin-like glycoproteins (PAS) in chronic gastric ulcer induced by 80% acetic acid in rats. Representative images of groups orally treated with vehicle (water, 1 mL/kg, Panels A and E), omeprazole (40 mg/kg, Panels B and F), sucralfate (100 mg/kg, Panels C and G) or SDI (191 mg/kg, Panels D and H) twice daily for five days after the gastric ulcer induction. Magnification = 20X, bars= 500 µm (Panels A-D), Magnification = 400X, bars= 50 µm (Panels E-H).

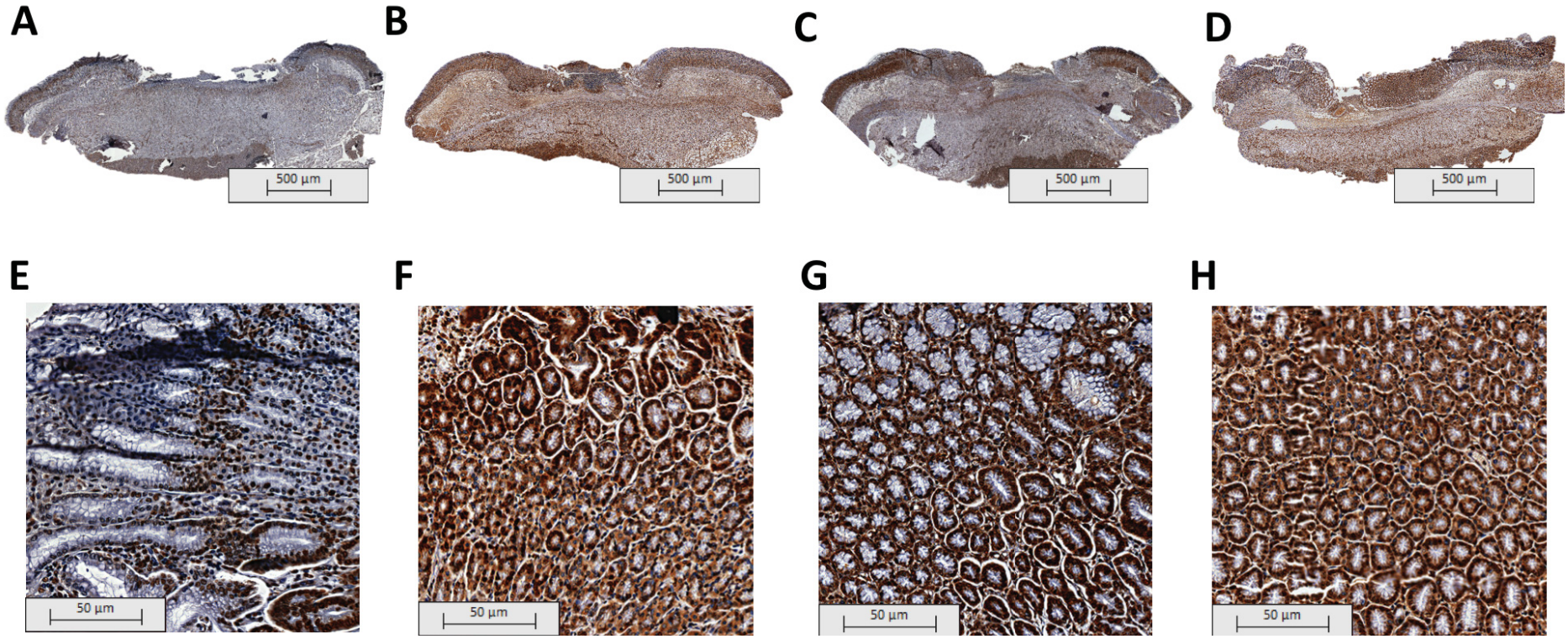


Fig. 4. Effect of *Sedum dendroideum* leaves infusion (SDI) on the immunohistochemical staining for PCNA in chronic gastric ulcer induced by 80% acetic acid in rats. Representative images of groups orally treated with vehicle (water, 1 mL/kg, Panels A and E), omeprazole (40 mg/kg, Panels B and F), sucralfate (100 mg/kg, Panels C and G) or SDI (191 mg/kg, Panels D and H) twice daily for five days after the gastric ulcer induction. Magnification = 20X, bars= 500 µm (Panels A-D), Magnification = 400X, bars= 50 µm (Panels E-H).

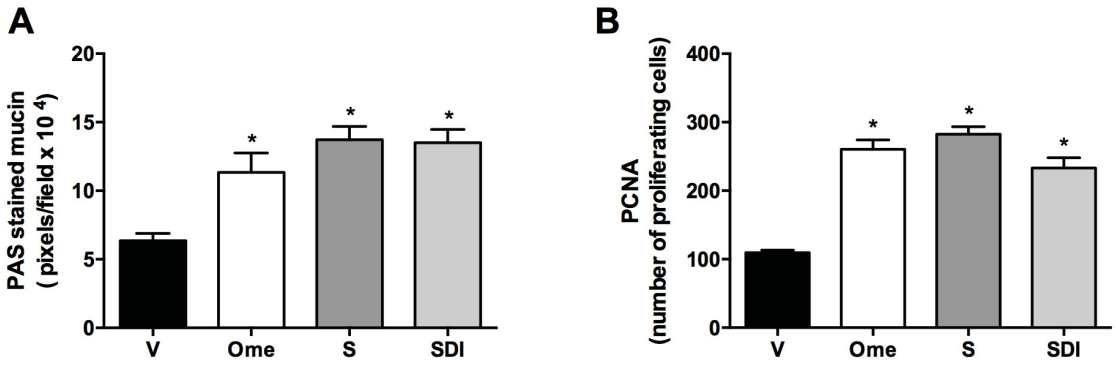


Fig. 5. Quantification of immunohistochemical staining for PAS (Panel A) and PCNA (Panel B) in chronic gastric ulcer induced by 80% acetic acid in rats. The animals were orally treated with vehicle (V: water, 1 mL/kg), omeprazole (Ome: 40 mg/kg), sucralfate (S: 100 mg/kg) or SDI (191 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n = 9). ANOVA followed by Bonferroni's test. *P<0.05 when compared to the ulcerated vehicle group (V).

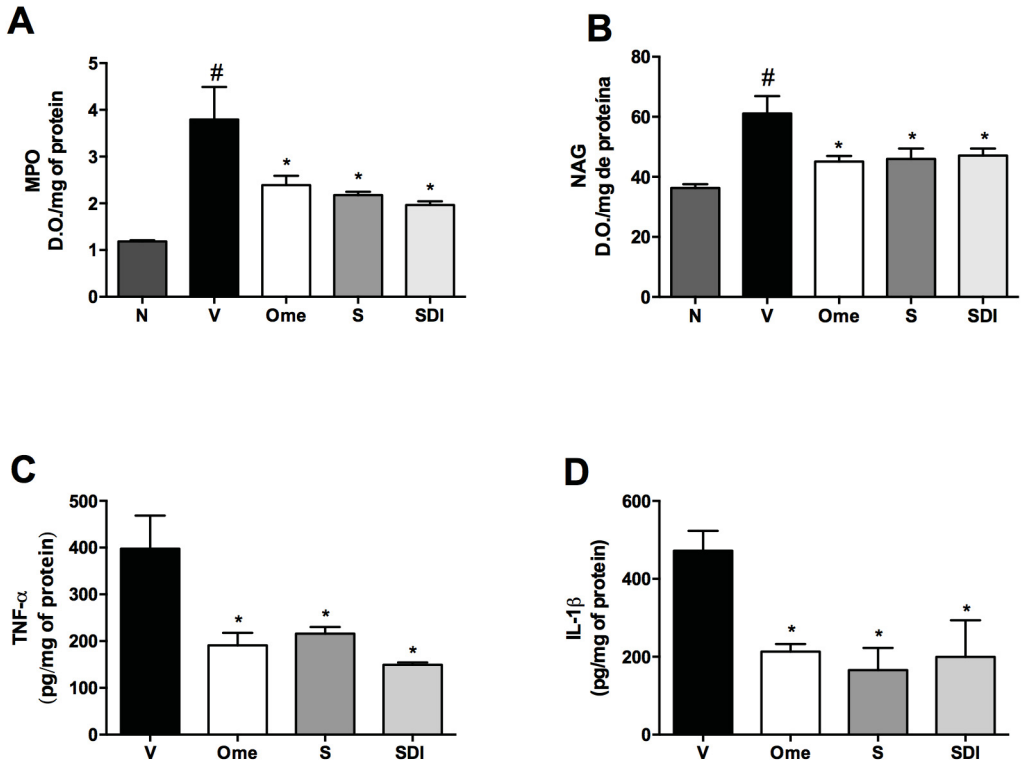


Fig. 6. Effect of *Sedum dendroideum* leaves infusion (SDI) on inflammatory parameters in chronic gastric ulcer induced by 80% acetic acid in rats. MPO activity (Panel A), NAG activity; (Panel B), TNF-α (Panel C) and IL-1β (Panel D) levels. The animals were orally treated with vehicle (V: water, 1 mL/kg), omeprazole (Ome: 40 mg/kg), sucralfate (S: 100 mg/kg) or SDI (191 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n = 6 - 3). ANOVA followed by Bonferroni's test. *P<0.05 and #P < 0.05 when compared to the corresponding value of the ulcerated vehicle group (V) or naïve group (N).

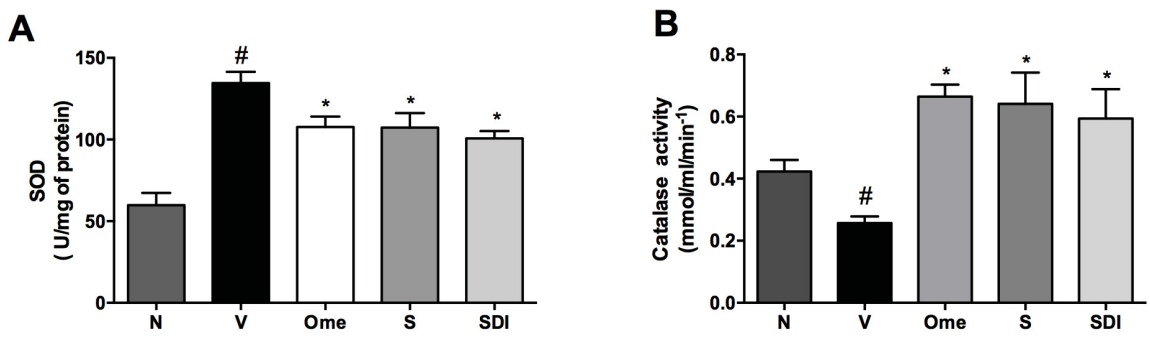
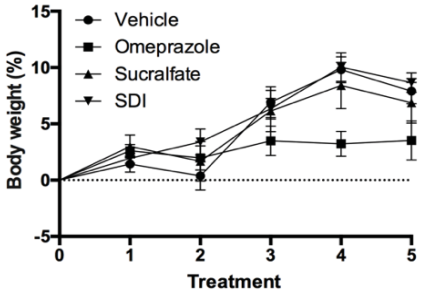


Fig. 7. Effect of *Sedum dendroideum* leaves infusion (SDI) on antioxidants parameters, represented by SOD (Panel A) and CAT (Panel B) activity in chronic gastric ulcer induced by 80% acetic acid in rats. The animals were orally treated with vehicle (V: water, 1 mL/kg), omeprazole (Ome: 40 mg/kg), sucralfate (S: 100 mg/kg) or SDI (191 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n = 5). ANOVA followed by Bonferroni's test. *P<0.05 and #P < 0.05 when compared to the corresponding value of the ulcerated vehicle group (V) or naïve group (N).

A



B

Table 1. Effect of *Sedum dendroideum* infusion on organ weights (organ weight/body weight) x 100)

Treatment	Adrenals	Spleen	Heart	Ovaries	Kidneys	Uterus
Vehicle (V: water 1 ml/kg)	0.021 ± 0.001	0.183 ± 0.010	0.260 ± 0.007	0.021 ± 0.002	0.518 ± 0.006	0.091 ± 0.016
Omeprazole (Ome: 40 mg/kg)	0.022 ± 0.002	0.194 ± 0.011	0.289 ± 0.003	0.024 ± 0.001	0.513 ± 0.014	0.106 ± 0.019
Sucralfate (S: 100 mg/kg)	0.019 ± 0.00	0.192 ± 0.006	0.290 ± 0.009	0.027 ± 0.001	0.478 ± 0.024	0.113 ± 0.031
Sedum dendroideum infusion (SDI: 191 mg/kg)	0.020 ± 0.00	0.175 ± 0.013	0.267 ± 0.014	0.020 ± 0.003	0.524 ± 0.017	0.098 ± 0.030

C

Table 2. Effect of *Sedum dendroideum* infusion on biochemical parameters

Treatment	AST	ALT	Creatinina	Urea
Vehicle (V: water 1 ml/kg)	160.95 ± 15.34	63.41 ± 2.44	42.20 ± 0.0	13.43 ± 0.37
Omeprazole (Ome: 40 mg/kg)	178.75 ± 14.85	48.14 ± 4.77	40.24 ± 1.16	14.04 ± 0.81
Sucralfate (S: 100 mg/kg)	110.14 ± 12.93	38.72 ± 2.44	38.29 ± 1.38	14.01 ± 1.30
Sedum dendroideum infusion (SDI: 191 mg/kg)	164.11 ± 37.93	45.01 ± 5.96	37.02 ± 2.17	12.87 ± 0.55

Fig. 8. Effect of *Sedum dendroideum* leaves infusion (SDI) on the % of body weight (Panel A), relative organ to body weight ratio (Panel B) and on serum biochemical parameters (AST, ALT, creatinine and urea levels, Panel C). The animals were orally treated with vehicle (V: water, 1 mL/kg), omeprazole (Ome: 40 mg/kg), sucralfate (S: 100 mg/kg) or SDI (191 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n = 7 - 5). ANOVA followed by Bonferroni's test. *P<0.05 when compared to the ulcerated vehicle group (V).

5. CONSIDERAÇÕES FINAIS

O *Sedum dendroideum* é utilizado na medicina tradicional para o tratamento de inflamações, diabetes e desordens gástricas. Nossos resultados demonstraram que o infuso preparado com as folhas do *S. dendroideum* não apresentou toxicidade *in vitro* no ensaio de citotoxicidade MTT e durante o tratamento crônico em animais não foram observados sinais de toxicidade, alteração no peso corporal e de órgãos e nos parâmetros bioquímicos plasmáticos.

Em relação a atividade biológica, o SDI administrado por via oral apresentou atividade gastroprotetora nos modelos de úlceras induzidas por etanol e por indometacina, sendo que o efeito foi mantido quando o SDI foi administrado por via intraperitoneal. Até este ponto, nossos dados são semelhantes ao descrito por Carrasco e colaboradores (2014), que utilizaram um extrato hidroetanólico preparado com as folhas do *S. dendroideum*. No entanto, o tratamento intraduodenal com infuso não alterou a secreção ácida gástrica, como ocorre com o tratamento com omeprazol, droga antisecretora cujo mecanismo de ação se dá através da ligação irreversivel a bomba de prótons nos canalículos das células parietais da mucosa gástrica.

Atualmente, a supressão da secreção ácida gástrica é a terapia de escolha para o tratamento de desordens gástricas como refluxo gastroesofágico e úlceras gástricas. Entretanto, recentemente inúmeros efeitos adversos têm sido relatados devido a supressão ácida gástrica. Em 2017 foi publicada uma revisão na revista "Gastroenterology" listando as complicações da terapia de inibição das bombas de prótons, entre elas: anemia, osteoporose, infecções por *Clostridium difficile*, formação de pólipos na mucosa gástrica e aumento no risco de desenvolvimento de demência e problemas cardíacos (VAEZI; YANG; HOWDE, 2017). Além disso, uma meta-análise publicada em 2018, demonstrou que os efeitos adversos que antes eram vistos apenas com o uso a longo prazo dos inibidores da secreção, podem surgir com apenas 4 semanas de uso dessa classe de medicamento (NAITO *et al.*, 2018).

Levando em consideração que o SDI não altera a secreção ou acidez do suco gástrico e o extrato hidroetanólico utilizado por Carrasco (2014) aumenta o pH e diminui a acidez gástrica, podemos concluir que os efeitos benéficos promovidos pelo infuso são diferentes dos efeitos causados por outros extratos, possivelmente devido aos metabólitos que são extraídos pelos solventes. A caracterização bioquímica demonstrou que o SDI é rico em flavonoides como quercetina, miricetina, kaempferol e outros glicosídeos que

isolados demonstraram efeitos anti-inflamatório e anti-úlcera (ALKUSHI; ELSAWY, 2017; LI et al., 2018). Ademais, o infuso *per se* demonstrou atividade antioxidante no ensaio *in vitro* DPPH.

Em relação ao mecanismo de ação gastroprotetor do infuso, existe um aumento nos níveis de muco e GSH na mucosa gástrica, considerados a primeira linha de defesa contra agentes agressores no estômago. Levando em consideração que o SDI não altera a secreção gástrica, avaliamos o papel do óxido nítrico no efeito antiulcera do infuso. O óxido nítrico tem papel fundamental na proteção mucosa, sendo um potente vasodilatador, auxilia no aporte de oxigênio e remoção de substancias nocivas, entretanto quando utilizamos um bloqueador da síntese de óxido nítrico, o efeito gastroprotetor do SDI não é mais visto, confirmando que o efeito farmacológico é dependente de óxido nítrico.

Considerando os efeitos demonstrados em modelos de úlceras agudas, nós avaliamos o efeito do SDI em úlceras induzidas por ácido acético, modelo de úlcera crônica que mais se aproxima ao visto na clínica. Surpreendentemente, o tratamento com SDI por 5 dias foi capaz de acelerar a cicatrização da úlcera gástrica, diminuindo os parâmetros inflamatórios como infiltração celular e liberação de citocinas inflamatórias e reforçando a defesa antioxidante. Análises histológicas também demonstraram que o SDI também aumenta a proliferação celular e a marcação de mucina, fatores importantes para regeneração total do tecido.

Outro efeito demonstrado pelo SDI foi atividade pró-cinética, ou seja, o aumentou o peristaltismo intestinal, aumentando a distância percorrida pelo marcador no intestino dos animais que receberam SDI por via oral, porém sem alterar o esvaziamento gástrico.

Em conjunto, todos os dados acima listados demonstram que o infuso preparado com as folhas do *Sedum dendroideum* (SDI) como uma alternativa terapêutica para desordens do trato gastrointestinal como úlceras gástricas. A nova legislação de produtos fitoterápicos tradicionais poderia facilitar o registro de um infuso como o deste trabalho devido ao uso popular já descrito da planta, ou ainda estimular a entrada da planta na lista do RENAME. Além disso, em conjunto os resultados reforçam o uso etnofarmacológico do SDI para tratamento de úlceras gástricas, sem evidencia de toxicidade em modelos animais.

CONCLUSÕES

Os resultados obtidos demonstraram que:

• O SDI não demonstrou sinais de toxicidade em células;

• O tratamento crônico com SDI não causou alterações no comportamento, parâmetros bioquímicos ou alteração de peso dos animais;

• O SDI protege a mucosa do estômago contra lesões induzidas por etanol e por anti-inflamatórios quando administrado pela via oral ou intraperitoneal;

• O SDI protege a mucosa gástrica sem alterar a secreção gástrica, aumento os fatores de proteção do estômago como o muco gástrico e os níveis de GSH;

• O SDI protege a mucosa gástrica de forma dependente de óxido nítrico;

• O SDI foi capaz de acelerar a cicatrização de úlcera gástricas induzidas por ácido acético;

• O SDI diminuiu os parâmetros inflamatórios, como migração celular e citocinas pró-inflamatórias;

• O SDI acelera a regeneração celular através do aumento da proliferação celular e conteúdo de mucina na úlcera gástrica;

• O SDI também foi capaz de agir como defesa antioxidante, alterando os níveis da enzima superperóxido dismutase e catalase;

• O SDI tem atividade pró-cinética, sem alterar o esvaziamento gástrico;

• Validação do uso popular tradicional do *Sedum dendroideum* para o tratamento de úlceras gástricas.

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